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Plant-derived and *Streptomyces* bio-compounds for the sustainable management of fungal pathogens and weeds: *in vitro* and *in vivo* studies

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PART I

1. INTRDUCTION

1.1. The EU Regulatory Framework on Sustainability Regarding the Use of Plant Protection Products

Many countries have implemented policies to reduce the use of chemical inputs in agriculture based on environmental and human health concerns. The United Nations has called for global action to reduce reliance on synthetic fertilizers and pesticides as part of the Sustainable Development Goals, and numerous public policies have been implemented at the national or regional level in this regard (Brunelle et al., 2024). Each of the four largest agricultural producers (USA, EU, China, and Brazil), has different regulatory policies regarding the use of chemical pesticides. In the USA the pesticide industry only has to demonstrate that its products "*will not generally cause unreasonable adverse effects on the environment*," which is partially defined as "*any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide*…" (US Environmental Protection Agency, 2026). Risk is primarily associated with ingestion, since the safety threshold in setting food residue tolerances is set to a "*reasonable certainty of no harm*" for pesticide exposure to humans through food, water and home uses (US Environmental Protection Agency, 2025). However, potential risks to plants, animals, the broader environment, and to humans from occupational exposures remain subject solely to cost-benefit analysis (Donley, 2019). In China, pesticide regulation has suffered from scattered data, complex laws and lack of transparency regarding rule implementation and compliance (Snyder & Ni, 2017). Recently, China has passed modest regulations updating certain aspects of pesticide use in the country, including establishing licensing requirements for pesticide sellers, record keeping requirements for users, and committees in charge of evaluating pesticide safety. One notable area where China has progressed in recent years is with banning or phasing out highly hazardous pesticides. As of 2014, the Chinese Ministry of Agriculture (MOA), the lead pesticide regulatory agency. which upholds the newly revised Pesticide Management Law, had banned or was in the process of phasing out 50 pesticides and in the process of restricting another 30. More recent regulations have resulted in the announced phase-out of an additional 12 pesticides by 2022 (Donley, 2019). Brazil's pesticide regulations are overseen by three governmental agencies: the Ministry of Agriculture (MOA), the Health Regulatory Agency (ANVISA), and the Ministry of the Environment (MOE). Under Brazil's 1989 Pesticide Law No. 7802, the country incorporated a more protective "*hazard assessment*" by which it can ban carcinogenic, teratogenic, mutagenic and hormone disrupting pesticides (Pelaez et al., 2013). However multiple factors have severely limited the effectiveness of human and environmental health safeguards in Brazil, including: 1) barriers limiting how frequently pesticides

are re-evaluated; 2) the Brazilian MOA's aggressive protection of the agrochemical industry; and 3) massive budget and personnel shortfalls (Garcia et al., 2005; Pelaez et al., 2013). Despite this, ANVISA and the MOE have been effective in getting some hazardous pesticides banned in the country (Donley, 2019).

The European Union has the most comprehensive and protective pesticide regulations of any major agricultural producer. Indeed, the EU has one of the world's strictest agricultural chemical regulations. The regulation of pesticides in the European Union (EU) relies on a network of hard law (legislation and implementing acts) and soft law (non-legally binding guidance documents and administrative and scientific practices). Both hard and soft laws govern how risk assessments are conducted, but a significant role is left to the latter. The first regulation that attempted to harmonize the evaluation of Plant Protection Product (PPP) was Directive 91/414/EEC. Before this Directive, each country of the EU had its own list of approved and banned active substances (AS), Having different lists for each country led to complications in treading food products across the EU, as an AS approved and used in one country could be banned in another. Directive 91/414/EEC introduced the two-tier system, which still represents the first pillar of the Regulation (EC) No 1107/2009 today. By this system the European Commission, supported by the European Food Safety Authority (EFSA), evaluates and approves the ASs, while each member country authorises the commercial product based on that specific AS. Directive 91/414/EEC was later replaced by Regulation (EC) No 1107/2009, due to problems related to risk evaluation and logistical and legal challenges. Previously, the safety risk of each AS was associated more with farmer' exposure than with its toxicological characteristics. Although toxic, a specific AS could still be used if the operator wore appropriate protection or used it in low quantities. Regarding the logistical problems, Directive 91/414/EEC, not being a Regulation, had to be transposed by each country individually into national laws. Because over 1,000 older ASs were on the market when the Directive was enacted, the risk evaluation program for each country took nearly 15 years (Bozzini, 2017). Regulation (EC) No 1107/2009 is the main piece of legislation governing PPPs and Ass since 2009. The Regulation aims to ensure agricultural productivity while preventing any negative effects on human and animal health, and the environment, resulting from the application of PPPs. Regulation (EU) No 1107/2009 is built on three pillars. The first pillar is the two-tier system. The second pillar is the "*Cut-off*" Criteria and Precautionary Principle, which introduces strict hazard rules. Any AS with proven negative effect on human health or the environment (carcinogenic, mutagenic, toxic for reproduction, or endocrine disruptor) is automatically banned regardless of the exposure levels. The final pillar is based on the promotion of biocontrol by reducing the time needed for the approval of low-risk products, and increasing their authorisation periods from 10 to 15 years. This same pillar mandates the gradual phase-out of high-

risk chemicals. Under Regulation (EC) No 1107/2009, active substances are continuously evaluated, resulting in the banning of hundreds of highly hazardous pesticides. Key chemical pesticides banned or not renewed for use in the EU agricultural sector include insecticides (chlorpyrifos and chlorpyrifos-methyl, imidacloprid, clothianidin, thiamethoxam, dimethoate, bifenthrin, endosulfan, lindane), herbicides (paraquat, atrazine, alachlor, butachlor) and fungicides (chlorothalonil, carbendazim, epoxiconazole, phosmet). These products have been banned due to concerns regarding their neurotoxic, reprotoxic, mutagenic and carcinogenic effects on operators and consumers, as well as their bioaccumulation, and negative impacts on aquatic wildlife and pollinator populations (Bozzini, 2017; European Commission, 2023). Implementing Regulation (EU) No 540/2011 is the main companion to the PPP regulation as regards the list of approved active substances. This Regulation transitioned all the substances that were deemed safe under older laws, specifically Directive 91/414/EEC, into the new system, and establishes a single database structure that constantly tracks which pesticides get approved by the EU and which get banned. All the information on a given active substance is centralized in this pesticide database, including Review Reports that contain the Good Agricultural Practices (GAP) usage tables (Bozzini, 2017). Furthermore, Regulation (EC) No. 396/2005 governs the rules on maximum residue levels (MRLs) of pesticides in or on food as well as plant and animal feed. Indeed, while the previous regulations focused on which pesticide could be used in agriculture, this regulation focuses primarily on consumers. The MRL is the highest concentration of a pesticide allowed for marketing and consumption of any food. Although it standardizes the trade of food products, this limit is not a toxicity ceiling. The threshold is hundreds of times lower than the actual limit posing a risk to human health. Exceeding MRLs doesn't mean that food products represent a risk for consumers; rather, it prevents farmers from using doses of risky pesticides. The Regulation is based on two key pillars. The first is the default limit of 0.01 mg/kg applied to all the pesticides not explicitly mentioned in the legislation, as this is the lowest concentration detectable by analytical laboratory equipment. The second pillar regards the importation of food products from non-EU countries. The MRLs imposed on food products imported and sold in the EU market are the same as those applied to locally grown products, ensuring safety and preventing any unfair competition. If a foreign crop requires specific chemical pesticides to survive a climate or pest not present in the EU, the importing country must formally apply for an "Import Tolerance". In this case, EFSA allows the marketing of the specific food product only if the concentration of the AS doesn't exceed the established safety limits for human health (Bozzini, 2017). Finally, Directive No 2009/128/EC, known as the Sustainable Use of Pesticides Directive (SUD), established a regulatory framework for community action to achieve a reduction in the environmental footprint of the EU's food system. Operating outside the approval and registration procedure, it

encompasses all relevant National Action Plans (NAP), training, the sales of pesticides, public awareness-raising, pesticides storage, application and equipment (including the inspection of equipment in use), specific practices and uses (such as aerial spraying), as well as indicators and reporting on pesticide uses. Every EU country is legally required to draft and regularly update its NAP. SUD establishes that each professional worker involved in the commercialization and use of pesticides must undergo mandatory training, and makes Integrated Pest Management (IPM) legally obligatory for all professional farmers (Bozzini, 2017). IPM and organic farming, with reduced and no synthetic pesticide input, respectively, are considered to be adequate strategies to follow towards achieving sustainable food production (Eyhorn et al., 2019). All the Pesticide Application Equipment (PAE) must be periodically inspected in order to prevent potentially incorrect and health-hazardous application of pesticides. Moreover, the Directive divides the application area of chemicals into two different zones to prevent environmental contamination and preserve human health: 1) buffer zones, which are unsprayed strips near lakes and rivers to prevent their contamination due to wind drift; 2) sensitive areas, such as public spaces, school, playgrounds, and parks, in which pesticide application is heavily restricted or totally banned. In addition, the application of aerial pesticides by airplanes or helicopters is prohibited across the EU, with rare exceptions granted only under tightly controlled municipal permits. However, these regulations didn't achieve their intended outcomes in the European Union or in the countries that export food to Europe (Bozzini, 2017). While pesticide risk reductions are generally the result of restrictions on the use of hazardous pesticides, significant pesticide use reduction at the farm level are usually the result of the adoption of a new farming strategy with lower and more regulated pesticide use (Lamichhane et al., 2016).

The recently published EC "*Farm to Fork Strategy*" (European Commission, 2020), part of the "*European Green Deal*", sets the first pesticide reduction targets at the EU level: a 50% reduction in overall use and risks from chemical pesticides by 2030, which includes a 50% reduction in the use of the more hazardous pesticides by 2030. The targets will be achieved by measuring the quantity of active substances on the market, their hazard properties, and the sales of the more hazardous pesticides (European Commission, 2020). Moreover, there is currently an increasing societal and political shift towards more sustainable agricultural systems to balance both food production and ecological biodiversity levels (Petit et al., 2015). This shift is also promoted by economic incentives for farmers. In fact, the Common Agricultural Policy (CAP) for 2023-2027 heavily shifts economic support toward sustainable agriculture through "eco-schemes" and enhanced environmental conditions, providing economic relief to eco-friendly farmers. Eco-schemes are a new tool under the CAP that offer annual per-hectare payments to farmers who adopt agricultural practices beneficial to the environment and climate. In Italy, they are part of the National Strategic Plan (PSN) and absorb

roughly 25% of the CAP's first pillar funds (amounting to over €900 million per year) (Zappitelli, 2025). In addition to the government, also the consumers are now demanding for a more sustainable and less dangerous agriculture. In the last twenty years, the sales of organic food have crossed USD 90 billion (Willer & Lernoud, 2020). To encourage more frequent purchases of organic food, consumers' motivation may be stimulated by emphasizing ethical, green, health, and social values (Tandon et al., 2020).

1.2. Traditional Phytosanitary Management - Conventional Agriculture

Agricultural fields are natural ecosystems based on several relationships between plants, macro- and microorganism. These relationships consist mainly of allelopathic, parasitic, or symbiotic interactions. During their growth process, plants are subjected to long-term biotic stresses from fungal pathogens, weeds, insects, rodents, and nematodes, which cause significant changes in the structure and function of the ecosystems and often lead to yield losses. There are about 10,000 species of insects, 1,800 species of weeds, 1,500 species of plant diseases, 1,500 species of plant viruses and some species of rodents and nematodes that endanger crops. For thousands of years, plant pests have represented an important problem for the history of human survival and development. Plant protection is based on three main elements: 1) plant target recognition; 2) plant protection methods; and 3) plant protection machinery (Zheng & Xu, 2023).

The 19th century represents a turning point in the history of pesticides, as various efforts were made to develop new equipment. In the 20th century, efforts were focused on two main areas, the use of extracts from plants and certain inorganic chemicals as pesticides. Six types of plant protection methods have been developed, including chemical methods, biological methods, integrated pest management (IPM), agricultural methods, physical methods, and plant quarantine (Zheng et al., 2006). Between the 1940s and the 1980s, research led to an exponential increase of knowledge in agricultural science, known as the "Green Revolution". This revolution influenced all aspects related to crops, especially those regarding their phytosanitary management. In the 1950s and 1960s, the Green Revolution developed high-yielding crops in an effort to feed hungry people, but these crops had to be accompanied by pesticides, agrochemicals and artificial fertilizers (Zheng & Xu, 2023). In fact, the global increasing demand for high-quality and high-quantity food production has led to a 15-20-fold increase in the use of pesticides in order to enhance the availability of crop yields across the globe (Dassanayake et al., 2021). Pesticides can be considered as any plant, or microorganism that can affect food, health or comfort. The Environmental Protection Agency (EPA) defines pesticides as a class of chemical compounds used for the management of the population of pests, nematodes, and arthropods, which ruin our food sources and lead to a variety of health issues (Kaur et al., 2024). They

are generally applied to a wide range of crops to protect them against insects, weeds, and pests to ensure and improve crop yield (González García et al., 2022; Rani et al., 2021). Through competition for light, water, and nutrients (weeds), or the destruction of plant tissue (pests and pathogens), harmful organisms can potentially reduce crop yield by 10% to 40 % on average (Fried et al., 2017). The massive use of chemicals during the Green Revolution represents one of its main downsides. Farmers and companies lost interest in using and producing alternative products to chemical pesticides, making new crop varieties dependent on pesticides and fertilizers. The introduction of highly hazardous pesticides had immediate repercussions, such as poisoning deaths, especially in poor rural households that were totally unequipped to use or store chemicals properly. Moreover, the easy availability of these products increased the number of suicides by self-poisoning (Karunarathne et al., 2020). Pesticide suicide is estimated to have killed many millions of people over the last 50-60 years. In 1990, the World Health Organization (WHO) estimated that 200,000 pesticide suicides occurred each year, while recent studies estimate, considering the underestimation of pesticide suicides in India, 372,000 deaths/year in the 1990s and 168,000 deaths/year in the 2010s (Gunnel et al., 2007; Mew et al., 2017; World Health Organization & United Nations Environment Programme, 1990). Pesticide risk to human health is associated not only with incorrect storage, but also with exposure through contact with the skin, ingestion, or inhalation. The negative effects change based on the type of pesticide, the route of exposure, the duration of exposure, and the individual's health status. Within the human or animal body, synthetic chemical molecules can be metabolized, excreted, stored, or bioaccumulated in body fat (Pirsaheb et al., 2015; World Health Organization & United Nations Environment Programme, 1990). Among pesticides, insecticides (29.5% of global use) are the most dangerous, followed by fungicides (17.5% of pesticides used worldwide) and herbicides (47.5% of pesticides used worldwide). Their level of danger depends primarily on solubility; water-soluble pesticides can contaminate underground water, rivers, and lakes, causing harm to aquatic organisms. Moreover, fat-soluble pesticides are absorbed in animal fatty tissues and enter the food chain, resulting in a high risk of exposure for humans as well (Dassanayake et al., 2021; Mahmood et al., 2016). The risk associated with chemicals also changes based on their physical type. There are four physical types of pesticide formulations: gas/vapor, liquid, gel/foam (hydrogels), and solid (nanoparticles). Gas and highly volatile liquids are the most threatening to human health due to exposure risks. Being easily inhalable, gas/vapor pesticides pass rapidly through the lung walls, entering the bloodstream. Moreover, they can spread via wind, covering wide areas, and being colourless and sometimes odourless, they are undetectable by the operator. Liquid pesticides represent a medium risk for human health and the environment. They are absorbed faster by the skin and eyes than solid pesticides and can also be ingested if confused with drinkable liquids or if they

contaminate drinkable water. Solid pesticides are the least dangerous, as they are absorbed by the skin slowly, although powdery pesticides can be easily inhaled (World Health Organization, 2020). Chemical exposures can lead to negative dermatological, gastrointestinal, neurological, carcinogenic, respiratory, reproductive, and endocrine effects. Pesticide residues can be detected in a wide range of common foods, drinks, and other meals. Peeling and washing fruit does not completely eliminate pesticide residues. Generally, the concentration of a single chemical pesticide residue doesn't exceed the legislatively determined safe levels. However, these limits may underestimate the real risk associated with the ingestion of contaminated food due to simultaneous exposure to two or more chemical substances, which may have synergistic effects. The wide range of chemical pesticides and their numerous combinations reveal the real complexity of the problem. In fact, the mixture of two or more chemicals with probably carcinogenic or endocrine-disrupting effects may produce unknown adverse health effects. After ingestion, pesticide residues can accumulate in the human body, such as in human breast milk, raising worries about fetal exposure and health impacts on offspring (Nicolopoulou-Stamati et al., 2016). The exposure to chemical pesticides can also affect neurological health. Organophosphorus (OP) compounds, which were largely used as pesticides during the past half century, are known to be associated with neurodegenerative and neurodevelopmental disorders, including Alzheimer's, Parkinson's, dementia, amyotrophic lateral sclerosis (ALS), attention deficit hyperactivity disorder (ADHD), and autism. The increase of these disorders is associated with the alteration of the function of a group of enzymes named cholinesterases (ChE), which are involved in the hydrolysis of acetylcholine back to choline. Acetylcholine is involved in the neurotransmission of cholinergic neurons by binding to their receptors. Then, it must be rapidly removed from the cleft within time limits of less than milliseconds in order to stop the lateral diffusion and frequent activation of adjoining receptors. This removal is efficiently accomplished by ChE. Thus, agents like OPs that block the function of the cholinesterase enzyme cause an accumulation of acetylcholine in the synaptic cleft and subsequent stimulation of cholinergic receptors. Indeed, the accumulation of acetylcholine is proven to be associated with Alzheimer's disease. In addition, experimental studies exploring the underlying mechanisms have provided some evidence for the involvement of cholinergic deficit, oxidative stress, neuro-inflammation, and epigenetic modifications as processes that are common to both the toxicity of OPs and the pathophysiology of the mentioned diseases. In addition, genetic mutations and polymorphisms of different variants of some genes like paraoxonase have been shown to be implicated in both susceptibilities to OP toxicity and neurological diseases (Mostafalou & Adbollahi, 2018). Other than human health, pesticides also have a serious negative impact on the environment. Indeed, the high quantity of chemical residues inside food is strictly linked to their bioaccumulation and biomagnification in soil, surface water, and groundwater. The

accumulation of chemical compounds in the soil affects micro- and macro-organisms, such as the microorganisms involved in the degradation of organic matter or the fixation of atmospheric nitrogen, mycorrhizal fungi involved in nutrient uptake, or earthworms involved in the fertility of the soil (Kelley & South, 1980; Lang & Cai, 2009). In fact, a lot of the active compounds used in traditional agriculture remain in the environment, as microorganisms don't produce the enzymes capable of breaking them down. Their residues negatively affect biodiversity, since their application can harm a diverse range of non-target species. The negative effects include limited or slow growth, infertility or reduction in reproduction, alteration of behaviour, and changes in other physiological biomarkers of non-target microorganisms, plants, and animals within aquatic and terrestrial systems. In some cases, chemicals formulated and applied against a specific group of organisms show negative effects also on organisms that are phylogenetically different; for example, insecticides with negative effects on amphibians or beneficial soil microorganisms. For instance, neonicotinoids, which are the most widely used active substances (AS) for insecticides, can persist and accumulate in the soil. Being water-soluble and systemic, they can leach into waterways and contaminate the aquifer, and can reach high concentrations in the nectar and pollen of treated crops. Reported levels in soils, waterways, field margin plants, and floral resources overlap substantially with concentrations that are sufficient to control pests in crops, and commonly exceed the LC50 (the concentration which kills 50% of individuals) for beneficial organisms. Concentrations in nectar and pollen in crops are sufficient to substantially impact colony reproduction in bumblebees, while the consumption of a small number of dressed seeds offers a route to direct mortality in birds and mammals, although they're less susceptible than arthropods (Goulson, 2013; Wan et al., 2025). Indeed, the uncontrolled use of pesticides has reduced the number of several terrestrial and aquatic animal and plant species, as well as rare species of birds, such as the bald eagle, peregrine falcon, and osprey, beneficial insects like the honeybee, and even algae involved in the production of oxygen in lakes and rivers (Helfrich, 2009; Mahmood et al., 2016; Majewski & Capel, 1995). Taking into consideration the health and environmental effects of chemical pesticides, new policies are switching the management of biotic stress from traditional agriculture, based on the application of synthetic products, to a modern agriculture focused on the use of sustainable and eco-friendly means.

1.3. Sustainable Phytosanitary Management - Integrated Pest Management and Biological Control

The concept of sustainable agriculture and development has gained prominence since the publication of the Brundtland Report in 1987 (Culleton et al., 1994). The shift to eco-friendly management has recently modified scientific questioning and brought up new research challenges for agro-ecological research (Petit et al., 2015). High sustainability systems can be taken as those that aim to make the

best use of environmental goods and services while not damaging these assets. The key principles for sustainability are:

- Integrated biological and ecological processes into food production processes;
- minimize the use of no renewable input that cause harm to the environment or to the health of farmers and consumers;
- make productive use of the knowledge and skill of farmers, thus improving their self-reliance and substituting human capital for costly external;
- make productive use of people's collective capacities to work together to solve common agricultural and natural resource problems, such as for pest management (Pretty, 2008).

Sustainable management could be divided in Integrated Pest Management (IPM) and biological control. The main difference between them is the degree of freedom in the application of chemical pesticide, which are allowed in IPM and prohibited in biological control. IPM is the model of crop protection that has prevailed since its creation in the late 1950s. According to FAO (2020), "*Integrated Pest Management (IPM) means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM promotes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms*". IPM, previously known as Integrated Control, contributed to improve crop protection. This sustainable management is based on five essential principles:1) potentially harmful species will continue to exist at tolerable levels of abundance; 2) the ecosystem is the management unit; 3) use of natural control agents is maximized; 4) any control procedure may produce unexpected and undesirable effects; 5) an interdisciplinary approach is fundamental (Deguine et al., 2021). In addition, the main guidelines are as follows: 1) analyse the pest status and establish economic thresholds; 2) devise schemes to lower equilibrium positions; 3) during emergency situations, seek remedial measures that cause minimum ecological disruption; 4) devise monitoring techniques. The goal of the economic threshold is to prevent a pest population from reaching the point where its damage causes economic losses (Deguine et al., 2021). This type of management is also characterized by a "*IPM pyramid*" which establishes a hierarchal order of prioritized practices to follow, in which the use of chemicals is the last one. Basically, IPM enforced promotes the application of natural and risk-free alternatives instead of chemical traditional pesticides, which are still allowed, but as last option. IPM-related legislation faces three common constraints. First, diverging IPM definitions and connotations complicate the formulation and interpretation of clear policies. Indeed, 67 different definitions were identified between 1959 and 2000. Second, where IPM is codified into legislation,

there are unintended effects as the pressure of those with economic interests in chemical pesticides leads to a light limitation of them. Third, governs are still concerned about the efficacy of sustainable alternatives, leading the attention to the possible negative effects and risks of their application on non-target organisms and environment. These concerns are often translated into excessive stringent regulatory processes that tend to obscure positive benefits and delay the implementation of, and farmer access to, science-based solutions, which are primarily aimed at alleviating the well-documented adverse ecological impact of conventional control practices. In general, IPM suffers from governs and systems, which provides inadequate support to farmers. Even in countries and geopolitical entities where IPM is mandatory, like EU, governs limit the effectiveness of IPM-based policies (Deguine et al., 2021).

Regarding biological control, or biocontrol, it could be defined as the application of living agents and virus, against dangerous organisms to provide human benefits. In the last years there have been an increasing interest from industries and legislations in biological control as the future paradigm for crop protection. The rapid growth of the interest in biocontrol, and corresponding growth in related research, led to its fragmentation into sub-disciplines, each with its specific divergent definition. Despite the divergent definitions, the general term biological control acquired positive connotation and interest in society and research. Like for IPM, the terminological misuse and confusion clearly hinder cross-disciplinary scientific coherence and productive communication between researchers, legislators, and the biocontrol industry, at both national and international levels in the authorization and implementation of biocontrol products. Such interactions are complicated by the frequent use of inconsistently defined terms like bioactive products, bioprotection, bioprotective, biopesticides, biofertilizers, and biostimulants. In some cases, researchers suggest that an organism could be its own biocontrol agent (BCA) by being intrinsically resistant to pest and pathogens. This definition doesn't align with the scientific community, as biocontrol is based on the interaction between a pest, a living BCA targeting that pest, and a human stakeholder that benefits from the pest control service provided by the BCA. Indeed, BCAs are organism capable of natural controlling plant pests, such as pathogens, weeds and insects. Historically, plants were not considered as potential BCA, although, from a conceptual point of view, they have all the characteristic to be involved in biocontrol process. However, the main Regulation regarding the definition of biological agriculture is the Regulation (EU) 2018/848, which defines it as a global system of farm management and production based on the interaction between the best environmental practices, high level of biodiversity, preservation of natural resources, animal wellness and use of natural substances and procedures (European Commission, 2018; Stenberg et al., 2021). As result of the increasing awareness by producer and consumers about environment and sustainability, various products with the prefix "bio-" have been

introduced on the market for the plant protection. Despite the prefix, some of these products contain plant derived and not-living AS. From a legislative point of view, there is a need to maintain a clear distinction between these categories, but this distinction is not required for their commercialization. To avoid any misunderstanding, the term bioprotection is widely used to refer to protection provided by all tools of biological origin for management of pests, pathogens, and weeds. In general, bioprotection agents should originate from nature or be nature-identical when synthesized and in general have a low impact on human health and the environment. Thus, the term bioprotection is the most suitable to define all the procedure that involves both BCAs and non-living natural-based substances (Stenberg et al., 2021). Biocontrol could be divided into four main categories based on the source of the BCAs and the human intervention. In case of native BCAs, the biocontrol could be: natural biological control, if the microorganism action is not influenced or enforced by human intervention; conservation biological control, if BCAs activity is stimulated by human to improve their pest control potential. In case the BCA is applied or added as not present in the ecosystem, biocontrol could be: classical biological control, if the BCA introduced is supposed to become permanently established; and augmentative biological control, if it is mainly intended to be temporarily established. Both may be used to refer to native or exotic organisms (Stenberg et al., 2021). Natural biological control has been widely used in the last 30 years, especially in entomological field. Regarding fungal pathogens and weed management, soil suppressiveness is a specific type of natural biological control. Soil suppressiveness is based on the effects of native microorganisms on the infections by soil-borne fungal pathogens and the germination of the seeds of weed. This type of biological control may involve or the soil microbiome or just few taxa. Instead, conservation biological control may be achieved by all the actions, from establishing flower strips that provide nectar, pollen, shelter, to introduce new prey for the parasitoids, that stimulate resident agents. Also soil suppressiveness may be achieved by interfering with soil microbiome. Like natural biological control, also classical biological control, has been heavenly used since the second half of the 19th century. Indeed, this type of control is well-established, well-defined, and well-documented. Recently, classical biological control has been abandoned by farmers has introducing exotic organisms may be a risk. The time window of microbial-based treatments in augmentative biological control may consist in a specific season or the life cycle of the crop, as after harvesting the organisms are supposed to die because of starvation, adverse climate conditions, and/or disinfection. BCAs time of intervention may change in case their efficacy is based only on their direct application (inundative biological control) or also on their reproduction (inoculative biological control). However, is very difficult to establish if the organisms will reproduce or not (Stenberg et al., 2021). The legislation regarding biocontrol changes based on the cited types, and changes for each country. Conservation

biological control is essentially unregulated, unless it involves regulated management practices or products, and is not dealt with further. Classical biological control is generally covered by legislation targeting environmental protection and risk assessment, especially regarding importation of exotic species and quarantine rules. Risk evaluation of classical biocontrol initiatives are strongly influenced by pest risk analysis, like determining whether an introduced agent may itself become a problematic pest. For augmentative biological control, regulatory conditions are entirely different for macro- and microorganisms. In the EU, there is no common regulatory framework for invertebrates (macroorganisms), but several member states have introduced national, mandatory pre-market authorization of invertebrate biocontrol agents. The rules regarding invertebrate biocontrol agents focus on environmental risks and have been integrated into specific, national, regulatory frameworks, and the term "biological control agents" may even appear in the title of regulatory documents. By contrast, rules concerning augmentative biological control with microorganisms are usually incorporated into pesticide regulations and pay attention to both environmental and human risks. Following established rationales for pesticides, such legislation does not view a microorganism primarily as a biocontrol agent, but as an AS of the pest control product. Data requirements and uniform principles for authorization of microbial PPP in the EU refer only once to biocontrol. Special attention should be paid to organisms used in biological control or in IPM. The reference to biological control in the legislation for PPP doesn't take into account the microorganism evaluation system reported in the same regulation, but the eventual negative effects that the imported microorganisms of the PPP may have on other non-target organisms. Similarly, in the USA, augmentative biological control is regulated following the same framework of pesticides, as, like the Europe framework, the legislation refers to the microorganisms as BCAs, but as microbial pesticides, which belong to biopesticides. In general, the Regulation (EU) 2021/1165 is the key regulation about the products and substances allowed to be used in organic farming and organic food processing. This regulation is divided into clean, restrictive lists of AS allowed under strict condition regarding plant protection, fertilizers and soil conditioners, animal feed, cleaning and disinfection, and food additives and processing aids (European Commission, 2021; Stenberg et al., 2021). Apart from BCAs, European legislation regards also all the organism involved indirectly in the protection of plants, like those that promote their growth. Regulation (EU) 2019/1009 covers these organisms as a type of biostiumulant. Organisms that increase the resistance of plants to abiotic stress are covered by the fertilizer regulation, while those that increase defence to biotic stress by PPP framework (Stenberg et al., 2021).

1.4. Conventional, Integrated, and Biological Pest Management in Italy

In the period after World War II, Italian agriculture was characterized by an important evolution in agronomic practices and cropping system policies, mostly related to technological development and numerous regulations aimed at human health and environmental protection. For instance, diflubenzuron (DFB), first commercialized in Italy in 1977, was increasingly used in agriculture against orchard pests after 1983. In 1986–1988, resistance to DFB was first found in crop pests in orchards across northern Italy. During the 1990s, resistance increased across Europe, and DFB use in orchards was reduced by the end of the 1990s and finally replaced by other active ingredients (Poretta et al., 2022). Several problems related to chemical pesticides have occurred throughout the history of the country. The most famous chemical accident in Italian history is known as the Seveso accident. On 10 July 1976, a chemical factory explosion near Seveso, Italy, exposed residents to high levels of 2,3,7,8-tetracholorodibenzo-p-dioxin (TCDD or dioxin), a by-product of the manufacturing of 2,4,5-trichlorophenol, a chemical fungicide and herbicide. TCDD is known to be a human carcinogen and a potent endocrine disruptor. A runaway chemical reaction after the explosion resulted in the release of an aerosol cloud that included sodium hydroxide, ethylene glycol, sodium trichlorophenate, and an estimated 15 to 30 kg of TCDD over an 18 km^2 area. Area residents in the path of the aerosol cloud developed nausea, headaches, and eye irritation, and 19 children were admitted to local hospitals with skin lesions. In the ensuing weeks, the area experienced high animal and plant mortality, and nearly 200 cases of chloracne were reported among residents, mostly among children. A study launched one month after the explosion revealed latent effects of the exposure to TCDD, including neurological problems, infections, dermatologic lesions, and adverse impacts on pregnancy and child health. Studies revealed that exposure to TCDD affected the health of Seveso's children by altering liver function, immune function, and tooth development, and affected adult health through infertility, endometriosis, fibromas, early menopause, and abortion. Chronic health outcomes among Seveso residents consisted of cancer, cardiovascular disease, diabetes and metabolic syndrome, alteration of bone and thyroid health, as well as immune and neurologic functions, leading to an increase in the mortality rate. High levels of TCDD contamination were detected also in liveborn children of women exposed to the 1976 Seveso explosion. The accident resulted in the establishment of Directive 82/501/EEC, known as the "*Seveso Directive*", implemented in Italy by Presidential Decree 175/88. The Directive regards the management of industries involved in the production of hazardous products (Eskenazi et al., 2018). Although being the most well-known case, the Seveso accident wasn't the only example of the negative risks associated with chemical pesticides. For instance, the Italian National Health Institute identified 2,108 accidental poisonings related to plant-protection products. About half occurred in agricultural workplaces. Most cases were mild, but some were severe and at

least one death was reported. Insecticides were the most common cause. Moreover, the uncontrolled application of pesticides led to the death of 35 bee colonies in Molise in 2023, and the contamination of the Po River. The multiple negative effects of chemical pesticides, combined with European Community legislation, promoted alternative and sustainable means in Italy.

In Italy, sales of PPPs fell by 18% between 2021–2023 and 2012–2014. The decline primarily interested fungicides, followed by herbicides. Moreover, the number of AS contained in PPPs decreased by 24%. At the same time, the use of organic AS increased by 133%. Compared to other EU countries, Italy is a leader in food safety, being one of the countries with the lowest presence of pesticide residues in food, with just 1% of foodstuffs showing phytosanitary product residues above permitted limits. Innovation in the field of pesticides has also progressed: according to the observatory's data, more than half of the active substances currently authorised in the EU have been introduced in the last ten years. In Italy alone, 85% of the pesticides on the market today were approved after 2010. In addition, in the period from January 2024 to October 2025, 38 new pesticides have been authorised in our country for use in organic farming, 17 more than in the same period of 2023–2024 (Cappellini, 2025). National policies strengthened the affirmation of biological control as a valid alternative to traditional pest management. Indeed, the new Italian PAN aims to reduce the application of chemical pesticides and increase the Utilized Agricultural Area (UAA) up to 40%. The planned strategy to reach these goals is based on IPM, agronomic practices, biological production and control, and low-risk phytosanitary products (Ministry of Environment and Energy Security, 2025). In general, biological agriculture proves to be from 22% to 35% more profitable than conventional agriculture. Organic farmers have from 7% to 13% higher labour costs due to mechanical pest and weed control, as well as the need for more human resources for crop monitoring. However, these high costs are balanced by lower costs for external inputs, such as the avoidance of chemical fertilizers, chemical herbicides, and fungicides. Although organic yields are from 10% to 18% lower than conventional ones, what ultimately makes organic farming profitable is the premium price paid for organic crops. In particular, organic Italian farmers are characterized by a higher share of revenues derived from diversified activities, lower current costs (-18.9%), higher value added (+11.5%), and higher net income (+15.6%) when compared to conventional farmers. These higher profits are reflected in an increase in both the number of organic farms and the agricultural area dedicated to organic farming. In 2019, there were over 329,000 organic farms and 13,799 thousand hectares of utilized area in the EU, representing a 30.1% and 45.1% increase compared to 2013, respectively. This growth contrasts with the general decline in the total number of farms occurring in most EU countries. Italy is the first European country in terms of organic farms (70,561 farms) and the third in terms of utilized area (1,933 thousand hectares), after Spain (2,355 thousand hectares)

and France (2,241 thousand hectares), representing 21.4% and 14.4% of the organic farms and utilized areas of the EU, respectively. From 2013, the number of organic farms and utilized area increased by 53.5% and 51.3%, respectively. Moreover, Italian organic farms are significantly bigger than conventional ones. The average surface of Italian organic farms is 28.2 hectares, compared to 8.5 hectares of conventional ones. In 2019, the organic UAA was concentrated primarily in the South and Islands region (1,193.0 thousand hectares), followed by the Centre (438.9 thousand hectares), the North-East (246.4 thousand hectares), and the North-West (115.1 thousand hectares). Interestingly, the South and Islands region organic UAA represents 19.1% of the regional UAA, the Centre organic UAA represents 20.9%, the North-East organic UAA represents 10.8%, and the North-West organic UAA represents 5.8%. Thus, although the South and Islands region has the largest organic UAA, biological practices affected more of the Centre UAA. The regions with the highest orientation toward organic farming are Calabria (33.6%), Sicily (26.4%), and the Autonomous Province of Bolzano (26.3%), while the less "virtuous" ones appear to be Piedmont (0.8%), Valle d'Aosta (4.9%), Lombardy (5.4%), and Friuli-Venezia Giulia (5.5%) (Gismondi, 2022). In addition, more than half of national farms (53.7%) adopted practices linked to the circular economy. These practices have been adopted mainly in the North-East (63.7%), followed by the North-West (56.7%), Centre (56.2%), Islands region (55.4%), and South (46.3%) (ISTAT, 2026).

Given the adverse effects and risks of chemical pesticides on human health and the environment, alongside the growing interest of consumers and agricultural producers in sustainable agriculture, it is essential to identify new and innovative biological and natural means for pest management in agriculture.

1.5. Evolution of Biological Control and Sustainable Means

The number, regulation, and efficacy of biological control have changed over time. Although having been used for centuries for controlling pests, the biopesticides market has been experiencing rapid growth in recent years. According to 17th century records, plant extracts like nicotine were largely used to control a wide range of pests, while the efficacy of the fungus *Beauveria bassiana* was first assessed in 1835. Moreover, the applicability of mineral oil for plant protection was cited in experiments during the 19th century. The rapid institutional expansion of agricultural research in the early 20th century led to an increase in studies involving biopesticides. For instance, *Bacillus thuringiensis* was first studied during those years, becoming the first and most used biopesticide. Other commercial successes from the 1980s and 1990s involved the use of *Agrobacterium radiobacter* to prevent crown gall on woody crops, as well as utilizing *Pseudomonas fluorescens* for the prevention of fire blight in orchards with streptomycin-resistant pathogen populations.

The increase in biopesticide adoption and the rapid expansion of organic agriculture during the past decade led to the development of new biopesticides since the mid-1990s. Over 100 active ingredients have been registered with the United States Environmental Protection Agency (USEPA) Biopesticides Division since 1995. The increasing cost of developing chemical pesticides, from 85 million to 280 million, could also have contributed to the rise in biopesticide registrations. Currently, biopesticides represent 5% of the total pesticide market, but their trend is growing three times faster than that of conventional products. The interest in biopesticides is growing stronger because of two major reasons: 1) biological products alone, or in combination with conventional products, have the potential to provide superior yield and quality; 2) the increasing strict limitations and regulations on chemical pesticide ASs. The market for biopesticides differs among the main food producers worldwide. For instance, over 200 biopesticides are currently sold in the US, while only 60 are sold in the EU. In general, more than 225 biopesticides are used across the world. The main countries involved in the application of biopesticides are the USA, Canada, and Mexico (45% of the total sold biopesticides), while Asia uses a very low quantity of biopesticides (5% of the total sold biopesticides). The biopesticide market's average annual growth is 17% since 2016. Primary growth factors affecting the biopesticides market include growth in crop production, ease of application, an increase in the need for organic food, and a growing preference for sustainable pest control methods. Although biopesticides currently account for approximately 2% of the plant protectants used globally, the growth rate indicates an increasing trend during the past two decades, wherein the global use of biopesticides is increasing steadily by 10% every year. Nowadays, sustainable agriculture is based on many techniques, such as the application of natural compounds or beneficial microorganisms. Natural compounds usually consist of Agricultural Wastes (AWs), non-product outputs of the production and processing of agricultural products that may contain beneficial compounds for pest management. In particular, AWs are residues from the growing and processing of raw agricultural products, such as leaves, peels, and the outputs of oil extraction. AWs production is increasing, especially in developing countries which are adopting intensified farming systems. It is estimated that about 998 million tonnes of agricultural wastes are produced yearly, of which 80% consists of organic wastes (Obi et al., 2016). Powders obtained from dry AWs, as well as their extracts, also known as green pesticides, and their essential oils, can be a good alternative to traditional chemical pesticides due to their safety, eco-friendly nature, and higher compatibility (Rahman et al., 2016). Their application as compounds for biopesticide development within a circular economy framework represents a valid alternative to traditional AWs management, enhancing their environmental potential while incorporating economic sustainability (Soares et al., 2026). The current methods for managing crop residues, such as burning, are not sustainable and pose environmental and health hazards due to the production of particulate

matter and gaseous pollutants (Rao et al., 2024). The high potential of AWs for the sustainable management of pests is linked to the wide range of secondary metabolites with antimicrobial activity produced by plants, which are classified as phytoanticipins and phytoalexins according to their biosynthesis and accumulation (Vergine et al., 2020). Phytoanticipins are always produced and stored by the plant in special tissues, while phytoalexin production is stimulated by the interaction with pests, especially pathogens. Both are involved in antioxidant, insecticidal, antibacterial, and antifungal activities. They are classified into chemically diverse groups such as steroidal glycoalkaloids (SG), essential oils (EO), cyanogenic glycosides, phenolic compounds (PC), glucosinolates (GLS), and terpenoids (Greco et al., 2026; Piasecka et al., 2015). Their mechanisms of action are mainly based on the inhibition of cell wall or protein synthesis, inducing leakage from the cells by tampering with the function of membranes, or interfering with intermediary metabolism or DNA/RNA synthesis/function. Moreover, the development of bacterial resistance toward natural plant products has been thus far documented in a very limited number of cases, unlike with chemical pesticides (Radulovic et al., 2013). Sustainable agriculture is also largely based on the application of microorganisms, specifically in biological control. Microorganisms can inhabit several zones within and around plants, especially the rhizosphere. Within the rhizosphere, intense interactions may occur among plants, soil, and microfauna owing to the high carbon content and energy in this zone (Elnahal et al., 2022). Bacteria are the main component of the microflora associated with plants (10^9 cells/gram of plant root tissue), followed by fungi (10^5-10^6 cells/gram of plant root tissue), algae (10^3 cells/gram of plant root tissue), and protozoa (10^2-10^3 cells/gram of plant root tissue) (Beattie, 2007; Vega, 2018). Other than living organisms, biocontrol relies also on viruses. In recent years, viruses have been largely used to combat pests directly, by infecting pathogenic pests, and indirectly, through cross-protection or by vaccinating crops (Stenberg et al., 2021). Three different categories have been established to determine the interactions between beneficial microorganisms and plants. The first is comprised of microorganisms responsible for plant nutrition that interact directly or indirectly with the plant, such as nitrogen-fixing bacteria. The second category consists of microorganisms directly acting on plant growth by producing plant hormones, solubilizing phosphates, increasing iron uptake, and/or producing volatile substances. The third category is the BCAs, which include organisms that indirectly stimulate plant growth by preventing the growth of plant pathogens (Fanning et al., 2018; Nihorimbere et al., 2011; Podile et al., 2007). The efficacy of BCAs is due to different mechanisms of action. BCAs protect the plant by producing metabolites toxic to pathogens, competing for nutrients and space, parasitizing pests, or increasing plant's resistance. Antibiosis is the main mechanism involved in biocontrol. The production of antibiotic or antifungal compounds is usually associated with the production of other secondary metabolites that interfere with pathogen growth

and activity (Ghorai et al., 2021), like lytic enzymes involved in the disruption of chitin, proteins, DNA, hemicelluloses, and cellulose (Heydari & Pessarakli, 2010). Other important secondary metabolites are volatile compounds (VOCs). Most VOCs are capable of inhibiting the growth of pathogens, but a few of them have also been reported to have allelopathic effects against weeds (Flores-Vargas & O'Hara, 2006; Zeller et al., 2007). VOCs confer resistance against biotic and abiotic stresses to plants by triggering induced systemic resistance (ISR). Triggering resistance in plants by using harmless microorganisms is a valid biocontrol mechanism, as it increases the defence also against dangerous organisms (Garbeva & Weisskopf, 2020; Stenberg et al., 2021). Semiochemical are another group of secondary metabolites produced by living organisms, like plants and yeasts, that can affect pests. These compounds have a wide range of effects, from manipulating pest behaviour to repelling them or deterring their oviposition, which all lead to a lower pest population or reductions in damage to focal crops (Stenberg et al., 2021). BCAs can also prevent infection from pathogens by competing for space and nutrients, or any limited resources. Antagonists are often better acclimatized to adverse environmental conditions, providing them with a competitive advantage over pathogens. In some cases, both competitors cause at least some harm to the host plant, but in such cases where the strongest competitor causes less harm and replaces a more harmful competitor, this mechanism can be acknowledged as a basis for biological control (Köhl et al., 2019; Stenberg et al., 2021). Although antibiosis is the main mechanism involved in protection against pathogens, the direct consumption of pests is considered the most important process reducing damage to plants in natural ecosystems. In particular, a parasitoid is an organism that spends its larval stage developing inside or on a single host, eventually killing it. Some fungi can penetrate the external cuticle of insects, causing infections, while others can even kill the host after ingestion. Regarding fungal pathogens, mycoparasitism is an interaction in which a BCA directly attacks and parasitizes the pathogen, thus killing it or its propagules, to absorb and digest its contents. This mechanism of action regards primarily fungal BCAs, but in some cases, bacterial BCAs have also shown parasitic behaviour. Other microorganisms switch to predatory behaviour only under nutrient-limited conditions (Palmieri et al., 2022; Stenberg et al., 2021).

Phytopathogenic fungi, bacteria, and viral trophic mechanisms may be used in the biocontrol of weeds. Also, larger animals, like herbivores, can act as BCAs if they suppress unwanted vegetation (Stenberg et al., 2021). Parasitoids may also provide biocontrol by altering and affecting their prey's behaviour. Non-consumptive effects can reduce the mobility, feeding, and reproduction of pests. The synergistic application of consumptive and non-consumptive biocontrol can optimize the effects of parasitoids (Stenberg et al., 2021). Sometimes, BCAs are also plant growth-promoting microorganisms (PGPMs). In this case, PGPMs that co-grow with a plant host can stimulate its

growth through direct and indirect mechanisms, increasing its resistance to pests and abiotic stresses and reducing the risk of infection. The mechanisms involved in plant-growth promotion include enhancing plant nutrient uptake and/or use efficiency and/or crop quality traits (Elnahal et al., 2022). Although BCAs may be PGPMs, PGPMs are not considered BCAs. Indeed, these microorganisms, as well as some plant-derived substances, are often referred to as biofertilizers, a sub-group of biostimulants. A plant biostimulant is defined as "*any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content*". Fertilizers and biostimulants are part of the same family, as they share similarities in helping plants to assimilate nutrients, boosting tolerance to abiotic stress factors, and increasing the quality of crops (Dara, 2019). Claiming that microorganisms promote the growth of the plant has sometimes been used to avoid strict regulatory restrictions on BCAs, since any microorganism with positive effects against pathogen infection should be registered as a microbial pesticide (Stenberg et al., 2021). In fact, biostimulant functionality and regulatory guidelines may not be as well-defined as those for biopesticides (Dara, 2019). Other organisms that are involved in biocontrol, but are not properly BCAs, are vectors of BCAs. These organisms, primarily nematodes, insects, and, in some cases, fungi, can transfer a BCA from one plant to another, carrying the organism to the site of activity (Stenberg et al., 2021).

1.5.1. AWs in Fungal Pathogen Management

Raw AWs and plant extract forms, including ethanol extracts and their fractions, resins, and essential oils, have been reported to have antifungal activity and show potential for the control of phytopathogenic fungi (Sales et al., 2016). A strategy to employ the antimicrobial effect of AWs is applying them without previous treatment for soil amendment (Voss et al., 2024). Yohalem et al. (2011) assessed the efficacy of post-extraction lavender (*Lavandula angustifolia* Miller) and lavandin (*Lavandula* × *intermedia* Emeric ex Loisel.) against *Verticillium dahliae* in open fields, achieving a large reduction in viable microsclerotia. Moreover, Pane et al. (2013) demonstrated that raw vegetable AWs suppress diseases caused by *Rhizoctonia solani* and *Sclerotinia minor*. Many fungal pathogens, like *Agroathelia rolfsii*, *Alternaria alternata*, *Fusarium oxysporum*, *Penicillium* spp., *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*, have been reported to be inhibited by *Citrus* spp. fruit peels and extracts, primarily due to their limonene content (Kato-Noguchi & Kato, 2025). Many plant extracts, such as neem (*Azadirachta indica* A.Juss.), garlic (*Allium sativum* L.), tobacco (*Nicotiana tabacum* L.), kappettiya (*Croton laccifer* L.), syringa (*Syringa vulgaris* L.), ginger (*Zingiber officinale* Roscoe), and others, are being utilized to control and manage pests or diseases of different plants (Rahman et al., 2016). Crude extracts obtained from garlic and onion (*Allium cepa* L.) show high

efficacy in inhibiting phytopathogenic fungi, such as *Colletotrichum acutatum*, by interfering with ergosterol synthesis and affecting cell wall integrity (Teixeira et al., 2023). Pomegranate (*Punica granatum* L.) peel and leaves contain considerable amounts of phenolic compounds, including hydrolyzable tannins (punicalin, pedunculagin, and punicalagin), xanthonoids (mangiferin), and flavonoids (catechin, epicatechin, and rutin). These compounds possess significant antioxidant, antibacterial, and antifungal activities. The methanol extract of pomegranate peel and leaves exhibits high inhibition of the mycelial growth of *Alternaria citri*, *Aspergillus niger*, *Botrytis cinerea*, *Penicillium digitatum*, *Penicillium italicum*, and *Fusarium sambucinum* (Elsherbiny et al., 2016; Nicosia et al., 2016; Salahvarzi et al., 2011).

Plant essential oils have attracted extensive attention from scholars due to their natural antifungal and anti-corrosion effects. Six natural plant essential oils-cinnamon (*Cinnamomum verum* J.St.-Hill.), oregano (*Origanum vulgare* L.), clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry), tea tree (*Melaleuca alternifolia* (Maiden & Betche)), pomelo peel (*Citrus maxima* (Burm.) Merr.), and rosemary (*Salvia rosmarinus* Spenn.) have high inhibitory activity against *B. cinerea* and *A. alternata* (Ji et al., 2024). Moreover, oregano essential oil has been found to be effective in inhibiting *R. solani* as well (Wu et al., 2023). Essential oil obtained from sweet orange (*Citrus sinensis* (L.) Osbeck) peel, rich in limonene, shows high antifungal activity against *A. niger*, *Aspergilus ochraceus*, *Fusarium* spp., and *Penicillium* spp. (Farouk et al., 2022). Key lime (*Citrus aurantifolia* (Christm.) Swingle), lemon (*Citrus limon* (L.) Osbeck), grapefruit (*Citrus paradisi* Macfad.), and sweet orange essential oils also show inhibitory activity against the plant pathogenic bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* var. *carotovora*, and the fungi *A. alternata*, *B. cinerea*, *F. oxysporum*, and *Fusarium solani*. For the latter, the oil caused a strong reduction in mycelial growth and spore germination (Badawy & Abdelgaleil, 2014).

1.5.2. AWs in Weed Management

Plants naturally compete with other plants for resources such as nutrients, water, and sunlight, often employing a mechanism called allelopathy. To reduce competition, they produce biochemicals, also known as allelochemicals, capable of influencing the growth, development, reproduction, and, subsequently, the survival of other species. Allelochemicals consist of various chemical families and are classified into the following 14 categories based on chemical similarity: water-soluble organic acids, straight-chain alcohols, aliphatic aldehydes, and ketones; simple unsaturated lactones; long-chain fatty acids and polyacetylenes; benzoquinone, anthraquinone, and complex quinones; simple phenols, benzoic acid and its derivatives; cinnamic acid and its derivatives; coumarin; flavonoids; tannins; terpenoids and steroids; amino acids and peptides; alkaloids and cyanohydrins; sulfides and

glucosinolates; and purines and nucleosides. Plant growth regulators, including salicylic acid, gibberellic acid, and ethylene, are also considered to be allelochemicals (Cheng & Cheng, 2015).

The allelopathic compounds created naturally by plants are still present in plant by-products; therefore, they can be used to inhibit the growth of unwanted weeds in agricultural fields (Voss et al., 2024). AWs can thus exploit the dual function of soil amendments and weed control agents. Nettle (*Urtica dioica* L.), broad bean (*Vicia faba* L.), coffee (*Coffea arabica* L.), and corn (*Zea mays* L.) have been shown to be effective in inhibiting weed growth (Lorenzo et al., 2022). Mallek et al. (2003) reported that the application of dried and milled crop residues of different *Allium* spp. inhibited the germination of weeds in a concentration-dependent manner. Limonene contained in *Citrus* spp. peel powder shows an allelopathic effect when applied directly to the surface of the soil, promoting, in some cases, the growth of the crop infested by weeds. The main weeds affected by *Citrus* raw AWs and extracts are smooth amaranth (*Amaranthus hybridus* L.), redroot amaranth (*Amaranthus retroflexus* L.), large crabgrass (*Digitaria sanguinalis* (L.) Scop.), wild poinsettia (*Euphorbia heterophylla* L.), bigleaf morning-glory (*Ipomoea grandifolia* (Dammer) O'Donell), Italian ryegrass (*Lolium multiflorum* Lam.), little mallow (*Malva parviflora* L.), timothy-grass (*Phleum pratense* L.), and common purslane (*Portulaca oleracea* L.) (Kato-Noguchi & Kato, 2025).

AWs crude extracts and essential oils exhibit inhibitory effects not only against fungal pathogens but also on weed germination and growth. Similar effects to those observed with *Citrus* spp. raw AWs are observed with their crude extracts and essential oils (Kato-Noguchi & Kato, 2025). Bergamot (*Citrus* x *bergamia* (Risso) Risso & Poit.) essential oil reduces seed germination and seedling growth in a wide range of weeds, like redroot amaranth, field bindweed (*Convolvulus arvensis* L.), and curly dock (*Rumex crispus* L.) (Bozhuyu, 2020). Moreover, bergamot VOCs contained in the essential oil show an allelopathic effect against several weed species, like wild mustard (*Sinapis arvensis* L.), annual ryegrass (*Lolium rigidum* Schreb.), and hop trefoil (*Trifolium campestre* Gaudin) (Ferreira et al., 2025).

Chicory (*Cichorium intybus* L.) root extracts displayed inhibitory effects on seed germination, as well as on the growth of roots and shoots, in both barnyard grass (*Echinochloa crus-galli* (L.) P.Beauv.) and redroot amaranth; similarly, neem oil extracted from *Azadirachta indica* seed waste was found to be an effective bioherbicide against coffee senna (*Senna occidentalis*), retarding its germination and altering the normal development of the weed (De Paulo et al., 2023; Wang et al., 2011). As with bergamot, pomegranate crude extract shows varied phytotoxic effects on weeds. Acetone and methanol extracts inhibit the seed germination and seedling growth of purple false brome (*Brachypodium distachyon* (L.) P.Beauv.) and pendant amaranth (*Amaranthus caudatus* L.).

Moreover, when seeds were treated with the dried extracts from acetone and methanol, their germination and growth were completely inhibited (Balah, 2016).

1.5.3. Biological control agents (BCAs) in Fungal Pathogen Management

Soil-borne fungal pathogens and oomycetes are notoriously difficult to control using traditional approaches, resistant varieties, and even chemical control (Palmieri et al., 2022). The soil-resident microbial community can suppress aggressive soil-borne fungal pathogens and oomycetes, such as *Fusarium* spp., *R. solani*, *Pythium* spp., *Phytophthora* spp., *Sclerotinia* spp., *A. rolfsii*, and *V. dahliae* (Ampt et al., 2019; Cacciola & Gullio, 2019). Manipulating the microbial composition of the soil can be useful for the control of pathogens that damage roots (Montesinos et al., 2009). *Trichoderma* spp. and their bioformulations are widely used for the control of soil-borne fungal pathogens due to their beneficial actions and the multitude of their mechanisms of action (Palmieri et al., 2022). Other fungi involved in the biocontrol of soil-borne fungal pathogens are those that form mycorrhizae, although they are less effective than *Trichoderma* spp. as their mechanisms of action are mainly based on the induction of resistance in the host plant and on improving its nutritional status (Bücking, 2012; Harrier & Watson, 2004). As with mycorrhizae, bacteria can also inhibit soil-borne fungal pathogens, primarily by promoting plant growth (Shaikh & Sayyed, 2014). The most studied species belong to the genera *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Burkholderia* (LeBlanc, 2021). Different bacterial and fungal species can also inhibit air-borne fungal pathogens. Microbial communities can inhabit both external surfaces (epiphytes) and internal plant tissues (endophytes), and these communities play an important role in protecting plants from diseases. *Ampelomyces quisqualis* is one of the best-known examples of a fungal biocontrol agent against air-borne diseases (Wilson, 1997). In particular, *Chaetomium* spp**.** and *Agroathelia bombacina* suppress *Venturia inaequalis*; *Rhodotorula kratochvilovae* strain LS11 controls *Monilinia* spp.; *Tuberculina maxima* parasitizes the white pine blister rust fungus *Cronartium ribicola*; *Darluca filum* and *Verticillium lecanii* parasitize several rusts; *Tilletiopsis* spp. parasitize the cucumber powdery mildew fungus *Sphaerotheca fuliginea*; and *Nectria inventa* and *Gonatobotrys simplex* parasitize two pathogenic species of *Alternaria* (Agrios, 2005). Among bacteria, *Bacillus* and *Pseudomonas* have proven to be effective against air-borne diseases through antibiosis and the induction of systemic resistance (Vorholt, 2012). In particular, *Bacillus* spp. are the most used BCAs and appear to be effective in a wide range of pathosystems (Khare et al., 2012). *Bacillus* spp. and *Pseudomonas* spp. effectively control *A. alternata*, *A. niger*, *B. cinerea*, *Penicillium expansum*, *Neofusicoccum parvum*, *Phaeomoniella chlamydospora*, and *Phaeoacremonium aleophilum* (Andreolli et al., 2019). Moreover, VOCs produced by *Pseudomonas* inhibit *Phytophthora infestans* (De Vrieze et al., 2015). In contrast to soil-

borne diseases, yeasts are able to control phyllosphere pathogens, like *Monilinia* spp. (De Curtis et al., 2019). The post-harvest stage is the highest-risk phase in the management of fruits and vegetables, as severe fungal pathogens, mostly wound pathogens, can compromise their shelf life. In this phase, yeasts are the main BCAs used, while among bacteria only *Bacillus subtilis* and *Pseudomonas syringae* are effective against post-harvest diseases. In fact, yeasts have proven to be effective against *B. cinerea*, *P. expansum*, *Rhizopus stolonifer*, etc. (Palmieri et al., 2022). Some of these yeasts have been isolated directly from the plant surface, like *Papiliotrema terrestris*, which shows both inhibitory activity and biosafety for human health and the environment (Palmieri et al., 2021; Palmieri et al., 2025).

1.5.4. Biological control agents (BCAs) in weed management

There has been a great number of naturally occurring fungal plant pathogens evaluated for possible use as potential biocontrol agents, but only a small proportion has been developed into commercial products (Aneja et al., 2017). Among fungi, *Colletotrichum* spp., *Chondrostereum* spp., *Phoma* spp., and *Sclerotinia* spp. are reported to control several weeds, like northern jointvetch (*Aeschynomene virginica* L.), lambsquarters (*Chenopodium album* L.), creeping thistle (*Cirsium arvense* (L.) Scop.), small mallow (*Microgloma pusilla* Sm.), hemp sesbania (*Sesbania exaltata* (Raf.) Cory), dandelion (*Taraxacum officinale* F.H. Wigg.), white clover (*Trifolium repens* L.), and spiny cocklebur (*Xanthium spinosum* L.). Most of the fungal biocontrol agents used as mycoherbicides have been found to be hemibiotrophs (Aneja et al., 2017; Harding & Raizada, 2015). Among the bacterial plant pathogens, *Xanthomonas campestris* and *P. fluorescens* have shown the potential to be developed as bioherbicides, inhibiting most of the species tested (Banowetz et al., 2008; Harding & Raizada, 2015). Some viruses have also been investigated for their potential to control invasive or undesirable weeds, like Araujia mosaic virus against moth plant (*Araujia hortorum* Brot.), Araujia mosaic virus (strain OBPV00) against tropical soda apple (*Solanum viarum* Dunal), and pepino mosaic virus (PepMV) or tobacco mild green mosaic tobamovirus (TMGMV) against European black nightshade (*Solanum nigrum* L.) (Harding & Raizada, 2015). The mode of action of each biocontrol agent is variable and may range from the release of simple compounds with allelopathic effects, like cyanide, organic acids, and secondary metabolites (such as the antibiotic 2,4-diacetylphloroglucinol), to plant growth regulators, such as auxins (indole-3-acetic acid and δ-aminolevulinic acid). Bacterial and fungal microbes also produce a wide array of phytotoxins that interfere with the metabolism of weed plants and cause plant mortality (Sindhu et al., 2018). In some cases, the production of allelopathic secondary metabolites is promoted by the interaction with weed roots, as seen in some *Pseudomonas* strains. The interaction between *Pseudomonas* strains and root exudates produced by wild oats (*Avena*

fatua L.), littleseed canary grass (*Phalaris minor* Retz.), and toothed dock (*Rumex dentatus* L.) results in the production of cyanide, *p*-coumaric acid (a photosynthesis inhibitor that impairs cytochrome *c* oxidase activity in plants), and cytolytic enzymes (which cause root cell wall degradation) in the weed rhizosphere (Dar et al., 2023).

Endophytic fungi also possess an extraordinary ability to produce volatile organic compounds (VOCs) with unique structural characteristics and broad biological activities. Fungi belonging to the genera *Muscodor*, *Xylaria*, and *Hypoxylon* produce VOCs that can inhibit a wide range of weeds (Macías-Rubalcava & Garrido-Santos, 2022). In particular, *Hypoxylon anthochroum* strain Blaci inhibits seed germination, root elongation, and seedling respiration of Prince-of-Wales feather (*Amaranthus hypochondriacus* L.), proso millet (*Panicum miliaceum* L.), red clover (*Trifolium pratense* L.), and alfalfa (*Medicago sativa* L.) (Ulloa-Benítez et al., 2016). Plant growth-promoting rhizobacteria (PGPR) may also be used for the management of weeds in combination with allelopathic bacteria. Indeed, this consortium provides effective control of weeds based on the allelopathic effect of the bacteria and the promoting effect of the PGPR on the crop (Cheng & Cheng, 2015).

1.6. *Streptomyces* genus in Fungal Pathogen and Weed Management

Streptomyces is a genus of Gram-positive, filamentous bacteria belonging to the group Actinomycetes. They are distributed in both aquatic and terrestrial environments, representing up to 90% of all soil Actinomycetes. They are ubiquitous soil bacteria, which are also found in marine environments, such as sediments. Some are symbionts of sponges, for example, or insects like the leafcutter ant *Acromyrmex octospinosus*, which lives in symbiosis with *Streptomyces* (specifically *Streptomyces* sp. S4). This bacterium produces antifungals that help protect the fungi cultivated by these ants from phytopathogens. *Streptomyces* has a particular development cycle. This cycle begins with a spore that germinates, forming vegetative hyphae with very few septa. These will structure into a network known as the vegetative mycelium, whose role is to explore the environment in search of nutrients. The bacterium will form compartmentalized aerial hyphae during nutrient deficiency; these hyphae will then differentiate into spores, which serve as the form of resistance and dissemination for this bacterium (Harir et al., 2018). This abundance is significant due to their role in producing bioactive compounds and supporting plant health and development (Nazari et al., 2022). Indeed, the genus *Streptomyces* is known for its capability to produce many biomolecules and bioactive secondary metabolites. *Streptomyces* species produce 70–80% of the natural bioactive substances known for their pharmaceutical or agrochemical applications. Continuously, new

metabolites with different biological activities are isolated from *Streptomyces* strains (Harir et al., 2018). It has been found that these metabolites can be broadly divided into four classes:

1. Regulatory compounds: these include growth factors, morphogenic agents, siderophores, and plant-growth-promoting rhizobia;
2. Antagonistic agents: these include antiprotozoans, antibacterials, antifungals, and antivirals;
3. Agrobiologicals: these include insecticides, pesticides, and herbicides;
4. Pharmacological agents: these include neurological agents, immunomodulators, antitumorals, and enzyme inhibitors.

In addition to secondary metabolites, *Streptomyces* produce medically and industrially important enzymes, such as uricase, L-asparaginase, cholesterol oxidase, cellulases, chitinases, chitosanases, amylase, proteases, and lipases (Harir et al., 2018). *Streptomycetes* have been the most frequently employed biological control agents in studies targeting *Fusarium* spp. The biocontrol activity against this genus is related to the production of secondary metabolites and VOCs. For instance, Chen et al. (2021) and Zhang et al. (2022) tested *Streptomyces alfalfae* and *Streptomyces malaysiensis* strains and obtained strong initial inhibitory responses against *F. oxysporum* f. sp. *vasinfectum* (on cotton) and *F. oxysporum* f. sp. *cubense* TR4 (on banana), respectively. They highlighted growth promotion, production of extracellular enzymes and metabolites, and pathogen suppression. *Streptomyces griseorubens* E44G and *Streptomyces* sp. FJAT-31547 effectively controlled *F. oxysporum* f. sp. *lycopersici*, also enhancing plant growth (Rashad et al., 2017; Zheng et al., 2019). Two marine *Streptomyces* strains, *Streptomyces* sp. (A19) and *Streptomyces griseoflavus* (A18), and two terrestrial strains, *Streptomyces* sp. (ED48) and *Streptomyces flavovirens*, successfully inhibited *F. solani*, partially through the production of VOCs (Phan et al., 2023; Torres-Rodriguez et al., 2022). Other fungal pathogens effectively controlled by *Streptomyces* spp. include *Alternaria* spp., *Botrytis* spp., *Colletotrichum* spp., *Magnaporthe* spp., *Phytophthora* spp., *Pythium* spp., *Rhizoctonia* spp., and *Agroathelia* spp. Against *A. rolfsii*, *Streptomyces* spp. enzyme suspensions hydrolyse cell walls, while solid formulations reduce sclerotia germination (Li et al., 2017). Instead, the inhibition performed against *Phytophthora* spp. and *Pythium* spp. involves mainly secondary metabolites, like indole-3-acetic acid (IAA) and staurosporine (Khucharoenphaisan et al., 2016; Wen et al., 2024). *Streptomyces* spp. inhibit *R. solani* on rice, soybean, and tomato by producing secondary metabolites, such as the 1,2,3-propanetriyl ester of dodecanoic acid, or by inducing specific defence-related responses in the plant, including increased activity of peroxidase (PO), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) (Singh et al., 2017).

Secondary metabolites of *Streptomyces* used as natural herbicides include anisomycin, bialaphos, carbocyclic coformycin, hydantocidin, herbicidines, herbumycin, phthoxazolin, and homoalanosin.

Anisomycin is an inhibitor of the growth of annual grassy weeds, such as barnyard grass and large crabgrass, as well as broad-leaved weeds, and it is produced by *Streptomyces* sp. Bialaphos, which is used for the control of both annual and perennial grassy weeds and broad-leaved weeds, is derived from *Streptomyces viridochromogenes*. Carbocyclic coformycin and hydantocidin are produced by *Streptomyces hygroscopicus* and serve to control several types of weeds. Herbicidines and herbumycins, isolated from *Streptomyces saganonensis*, are effective against monocotyledonous and dicotyledonous weeds. Finally, phthoxazolin and homoalanosin are produced by *Streptomyces* sp. and are also utilized for the control of several weeds (Harir et al., 2018). Moreover, different *Streptomyces* strains show high efficacy in inhibiting redroot amaranth, horseweed (*Conyza canadensis* L.), large crabgrass, common purslane, green foxtail (*Setaria viridis* (L.) P.Beauv.), and European black nightshade (Bo et al., 2019; Ferreira Junior et al., 2025; Ling et al., 2023). Other pests that can be controlled by the application of *Streptomyces* spp. are nematodes and insects. Actinomycetes were identified as antagonists of plant-parasitic nematodes. *Streptomyces* spp. that produce avermectins show high nematicidal activity. *Streptomyces avermitilis* produces ivermectin, which has efficient activity against *Wuchereria bancrofti*. Similarly, various other antiparasitic compounds are produced by various *Streptomyces* spp. Regarding insects, *Streptomyces* spp., *Streptosporangium* spp., and *Micropolyspora* spp. have great larvicidal activity against *Anopheles* mosquitoes and *Culex* larvae. The cell-free filtrate has remarkable larvicidal activity against *Aedes* and *Culex* vectors, which are involved in the transmission of dengue and filariasis. Some secondary metabolites produced by *Streptomyces* spp., like avermectins, flavonoids, macrotetrolides, and tetranectin, are classified as toxic to mosquitoes (Harir et al., 2018). Recently, *Streptomyces* has been used as an environmentally friendly "nanofactory" for developing clean and nontoxic procedures for the preparation of silver and gold nanoparticles (El-Naggar, 2024). The chemical techniques of nanoparticle preparation are less expensive when produced in high quantities; however, the nanoparticles may be contaminated by precursor chemicals, toxic solvents, and risky by-products. As a result, the development of high-yield, low-cost, non-toxic, and environmentally beneficial procedures for metallic nanoparticle synthesis, and thus the biological method of nanoparticle synthesis, is considered important. Actinobacteria are actually effective nanoparticle producers, showing a number of biological properties, including antibacterial, antifungal, anticancer, antibiofouling, antimalarial, antiparasitic, and antioxidant activities (Harir et al., 2018).

Although many studies have focused on the biocontrol activities of this genus due to its high production of bioactive compounds used as defence mechanisms, *Streptomyces* spp. also show a high capability in colonizing the inner tissues of host plants and promoting their growth (Sousa & Olivares, 2016). These attributes may be due to features such as quorum-sensing-controlled gene expression,

high multiplication rate, and the production of antibiotics, siderophores, cellulases, phytohormones, amino acids, chitinases, lipases, and beta-1,3-glucanase by plant growth-promoting *Streptomyces* (PGPS). For instance, *Streptomyces griseus* increases the growth of carrot (*Daucus carota* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), and wheat (*Triticum aestivum* L.). Another mechanism involved in the growth promotion of plants is the production of phytohormones. *Streptomyces coelicolor*, *Streptomyces exfoliatus*, *S. griseus*, *Streptomyces lividans*, *Streptomyces scabies*, and *Streptomyces violaceus* are reported to be auxin producers, which promote the growth and yield components (spikelet number, spike length, and fresh and dry mass of the developing grain) of wheat plants (Harir et al., 2018). *Streptomyces* spp. also have indirect effects on plant growth by modifying the soil microbiome. For instance, the recruitment of *Streptomyces* spp. to the rhizosphere is driven by the chemotactic movement of PGPMs (Olanrewaju & Babalola, 2019). Moreover, *Streptomyces* spp. and other genera belonging to Actinomycetes are involved in the bioremediation of toxic organic compounds and heavy metals. For instance, Actinomycetes can grow in oil-rich environments, and thus they can be used in the bioremediation of oil contaminants (Harir et al., 2018). In addition, *Streptomyces albus* showed the ability to degrade pyrethroid insecticides such as cyfluthrin, bifenthrin, and cypermethrin, with notable efficacy against deltamethrin (Chen et al., 2011).

1.7. Biological control and new challenges

The main limitation of biological control is that it is slower to suppress pest populations than most pesticides, as parasitized organisms may take several days to die; additionally, predators require a period of time to establish an economic level of pest suppression. Development costs of biological control are sometimes described as 'high', but these costs are much lower than those for the equivalent synthesis, toxicological evaluation, and marketing of a new pesticide, and substantial profits can be achieved from biocontrol with long-term, effective natural enemies. In biological control, pest populations are usually not eradicated but maintained at very low densities; thus, long-term suppression of pest species is the desirable aim. Eradication of a pest population after chemical treatment, even if achieved, occurs only on local scales, and the environment is then open to re-invasion, often resulting in a much-reduced natural enemy fauna.

It is now widely acknowledged that successful biological control depends on extensive preliminary studies to gain a comprehensive understanding of the biology and ecology of the pest and natural enemy complex, as well as of the environments from which they originate and have subsequently colonized, or into which they will be released. This research can be time-consuming, but it is essential and analogous to the period of time (estimated to be around 10 years) between the synthesis of a new molecule as a potential pesticide and placing the product on the market (Bale et al., 2008).

Novel techniques have been implemented in the selection process of BCAs to guarantee their efficacy. For instance, the rapid diffusion of next-generation sequencing (NGS) techniques has had a tremendous impact on the biocontrol field through the generation of whole-genome sequencing, transcriptomics (RNA-seq), and proteomics data. This allows for comparative genome analysis and gene/protein expression analyses to identify molecular pathways and key genes potentially playing a critical role in biocontrol. Several of these techniques have been applied alone or in combination to study the biocontrol and plant-promoting mechanisms of filamentous fungi. Transcriptomics (RNA-seq) could be used to identify genes involved in biocontrol and confirm their presence in potential BCAs, or to study how the genome expression of BCAs changes due to interactions with pathogens and the host plant. These techniques may be applied to fungi, bacteria, and yeasts with potential application as BCAs (Palmieri et al., 2022).

Although considered fundamental for the switch to sustainable management, only a few microbial biofungicides are currently available compared to conventional fungicides based on chemical active ingredients. Therefore, to implement effective disease management for many crops, there is an urgent need to develop other biocontrol products. From the laboratory stage, the development of a commercial microbe-based biopesticide consists of three complex phases:

1. Development of a viable and stable formulation;
2. Patent application;
3. Registration of the active ingredient and its formulation.

The formulation process is a key step for pesticide development in terms of production costs and effectiveness. To date, not much information is available on formulation processes and technologies, as they are often protected by trade secrets. A key point during the formulation process is to guarantee the viability of the BCAs, which is constantly stressed during this stage. To ensure the viability of BCAs, researchers should study the best processes with the lowest impact, as well as protectant and stabilizer compounds to add to the growth medium to guarantee their survival during storage. In addition, research should focus also on chemical additives or suitable packaging to preserve formulation stability and prevent contamination (Palmieri et al., 2022).

Another problem linked to biocontrol products regards their registration process. Indeed, there is often a lack of connection between basic research and industrial development, primarily due to the lack of protocols to evaluate the safety of BCAs for humans and the environment. Chemical pesticide registration provides all the protocols that can be used to ensure that they are not dangerous. The main problem is that these kinds of protocols are not applicable to BCAs, since they are based on the evaluation of the chemical AS of the PPP. The lack of knowledge regarding methods to evaluate BCA environmental safety could stretch the registration process. In the worst-case scenario, after the

evaluations have been completed, the BCA registration could be rejected, undoing the time and money spent through the efficacy evaluation and registration process. Thus, another important aspect that may concern researchers is the establishment of preliminary protocols to ensure that a BCA is not harmful to humans and the environment (Palmieri et al., 2025).

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PART II

2. AIMS AND OBJECTIVES

Plant pathogen, weed, and insect management is no longer solely based on the application of chemical pesticides. Traditional management relied heavily on pesticides to prevent yield losses due to biotic adversities. However, the overuse of agrochemicals, along with their negative impacts on human health, the environment, and biodiversity, and the subsequent rise of stricter regulations have become the driving forces behind discovering novel management strategies. As one of the main food producers worldwide, the European Union (EU) has the most robust regulatory framework for plant protection products (PPPs) and active substances (ASs). This framework primarily revolves around Regulation (EC) No 1107/2009, the milestone regulation that dictates which ASs are permitted. Since its implementation, this regulation has banned numerous chemical pesticides, effectively promoting sustainable management strategies that involve low or no chemical application. Integrated Pest Management (IPM) and biological control have emerged as the primary alternative strategies in modern agriculture:

- IPM combines natural, biological, and agronomic tools, treating traditional chemical products as a last resort;
- Biological control, by contrast, relies exclusively on plant-derived products, such as agricultural wastes (AWs), and biological control agents (BCAs).

Agricultural wastes (AWs), such as fruit peels from juice or essential oil production, leaves from pruning, and other plant parts, could be used as able to control effectively fungal pathogens, representing a valid alternative to traditional management. Moreover, because they are by-products, utilizing them in sustainable strategies perfectly aligns with the concept of the circular economy, thereby reducing waste management costs for farmers.

Consequently, the **first objective** of this thesis was to preliminarily evaluate the *in vitro* efficacy of 18 putative inhibiting powders (PIPs), obtained from 17 dried AWs, plus the commercial organic product EP5, against 31 fungal pathogens. This preliminary experiment was performed by incorporating the PIPs directly into the culture media at a concentration of 2% w/v, and then at 4% for those PIPs with lower efficacy (*Article 1, published*).

To address the fact that the Apulia region, and specifically the Foggia province, is one of the leading areas for the production of durum wheat (*Triticum durum* Desf.) and soft wheat (*Triticum aestivum* L.), the **second objective** of this thesis was formulated. Indeed, Apulia accounts for nearly 29% of the national cultivated area and 24% of total national production. However, this production is seriously threatened by soilborne fungal pathogens, such as *Fusarium algeriense*, *Fusarium culmorum*, and *Fusarium oxysporum* f. sp. *dianthi*, isolated from wheat root and collar, leaves and

kernels in an early study. Building upon the best-performing PIPs from the preliminary trial, this second aim was twofold:

- To evaluate the *in vitro* antifungal potential of seven PIPs against ten soilborne fungal pathogens;
- To assess their potential application for biofumigation *in vivo* against three specific soilborne fungal pathogens of wheat.

Once the most promising PIPs were identified *in vitro*, bergamot (*Citrus* × *bergamia* (Risso) Risso & Poit), pomegranate (*Punica granatum* L.), and the commercial product EP5 were selected for the *in vivo* greenhouse experiments against *F. algeriense*, *F. culmorum*, and *F. oxysporum* f.sp. *dianthi* (*Article 2, published*).

Moving beyond fungal pathogens, the **third objective** of the thesis took into account that the national production of durum wheat is heavily threatened by the development of weed biotypes exhibiting resistance to single or multiple herbicide mechanisms of action (MoA). This phenomenon is mainly documented in species such as corn poppy (*Papaver rhoeas* L.), sterile oat (*Avena sterilis* L.), and ryegrass (*Lolium* spp.), which are the most concerning weeds for Apulian production. To address this, water extracts (E) obtained from the powders (P) of bergamot, pomegranate, and EP5 were tested *in vitro* against black-grass (*Alopecurus myosuroides* Huds.), corn poppy, and Italian ryegrass (*Lolium multiflorum* Lam.). After evaluating the effects of these extracts on weed germination and early growth, a second experiment was conducted *in vivo* by applying the powders directly into the soil. This second trial was carried out under greenhouse conditions (*Article 3, submitted to* Frontiers in Agronomy *on 18 June 2026*).

Regarding the **fourth objective**, the study focused on another highly important crop for the Foggia province and the Apulia region: the tomato (*Solanum lycopersicum* L.). Apulian production represents 22.3% of the national total, with 19.4% concentrated specifically within the Foggia province. As observed for durum wheat, local yields can be severely affected by soilborne fungal pathogens, which can cause fruit losses ranging from 50% to 80%. In this study, we evaluated the efficacy of bergamot, pomegranate, and EP5 against *Athelia rolfsii* (syn. *Agroathelia rolfsii*), *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* under both *in vitro* and *in vivo* conditions. For the *in vivo* experiment, the three PIPs were applied at lower doses compared to the wheat trial, and their potential biofumigation effect was not evaluated (*Article 4, published*).

Sticking to tomato cultivation, **the fifth objective** of this thesis addressed weed management, as if not properly managed, tomato production can experience yield losses of up to 75% due to weed interference. Specifically, in the Apulia region, yield losses caused by weeds can reach up to 100%. The main local weeds include parasitic plants like broomrape (*Orobanche* spp.), broadleaf weeds like

black nightshade (*Solanum nigrum* L.) and pigweed (*Amaranthus* spp.), and grasses such as barnyardgrass (*Echinochloa crus-galli* [L.] P.Beauv.) and bristlegrass (*Setaria* spp.). The *in vivo* efficacy of bergamot, pomegranate, and EP5 powders was also tested against common tomato weeds, specifically redroot pigweed (*Amaranthus retroflexus* L.), common lambsquarters (*Chenopodium album* L.), and black nightshade (*Article 5, ready for submission to* Weed research).

In addition to plant-derived products, biological control relies on the application of living organisms, particularly microorganisms known as Biological Control Agents (BCAs). BCAs can suppress fungal pathogens, weeds, and insects through various mechanisms. Fungal BCAs act mainly via mycoparasitism or enzymatic degradation, whereas bacterial BCAs typically utilize root colonization and metabolite production. To expand on this approach, the **sixth objective** of this thesis focused on the use of actinobacteria. Within this context, *Streptomyces* spp. have recently shown high applicability in controlling fungal pathogens, especially soilborne ones, through direct or indirect mechanisms of action. Indeed, this genus is renowned for producing a wide range of secondary metabolites that can directly inhibit fungal growth or promote plant growth, thereby enhancing host resistance to infections. Based on this potential, three *Streptomyces* strains (Strep_22, Strep_23, and Strep_24) were selected to evaluate their inhibitory activity *in vitro* condition against ten soilborne fungal pathogens. Strep_22 emerged as the most effective strain; consequently, its efficacy was subsequently tested *in vivo* against the soilborne fungal pathogens of wheat and tomato. Moreover, its plant growth-promoting (PGP) traits were also evaluated (*Article 6, submitted to* Pest Management Science *on 02 June 2026*).

Finally, the **seventh and final objective** of this thesis focused on the molecular and metabolomic characterization of the most promising strain. *Streptomyces* spp. secondary metabolites are reported to have broad antifungal, antibacterial, larvicidal, insecticidal, nematicidal, and even antiviral applications. Their biocontrol efficacy is also linked to the production of lytic enzymes, volatile organic compounds (VOCs), and molecules that stimulate plant growth and defence pathways, such as siderophores and phytohormones. However, the molecular characterization of *Streptomyces* spp. is highly complex due to significant genomic differences among species. Even between closely related species, there is substantial variability in chromosomal protein-coding genes, species-level core genes, accessory genes, and genetic fingerprints. Notably, secondary metabolite biosynthetic gene clusters (smBGCs), carbohydrate-active enzymes (CAZymes), and protein-coding genes bearing the rare TTA codon demonstrate high intra- and inter-species variability. smBGCs are physically clustered groups of two or more genes in a genome that collectively encode the enzymes and pathways required to produce specific secondary metabolites. Therefore, it has been performed a comprehensive metabolomic and molecular identification of strain Strep_22. The specific aim was to

identify the specific metabolites involved in its inhibitory activity and plant growth-promoting traits, determine its taxonomic species, and link the produced metabolites to their respective smBGCs (*Article 7, ready for submission to* Biological Control).

In summary, this thesis aims to provide a comprehensive, innovative, and highly applicable toolkit of bio-based solutions (plant extracts and specialized microorganisms) ready to support the ecological transition of Mediterranean agri-food chains.

Objective 1. Preliminary evaluation the *in vitro* efficacy of 18 putative inhibiting powders (PIPs), obtained from 17 dried AWs, plus the commercial organic product EP5, against 31 fungal pathogens. This preliminary experiment was performed by incorporating the PIPs directly into the culture media at a concentration of 2% w/v, and then at 4% for those PIPs with lower efficacy:

Morea, M. G., Conte, T., Ricciardi, G., Raimondo, M. L., & Carlucci, A. (2025). Preliminary Study on the Antifungal Potential of Selected Plants as Botanical Fungicides Against Main Fungal Phytopathogens. Plants, 14(23), 3634. https://doi.org/10.3390/plants14233634

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Preliminary Study on the Antifungal Potential of Selected Plants as Botanical Fungicides Against Main Fungal Phytopathogens

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Abstract

Fungal diseases represent relevant constraints on global agricultural productivity, causing severe yield losses and deterioration of crop quality. The extensive use of chemical fungicides has produced environmental and health concerns due to their persistence, bioaccumulation, toxicity, and the increasing development of resistant fungal strains. To promote sustainable plant protection strategies, this study aimed to evaluate natural alternative products derived from botanical sources and agro-industrial wastes. Eighteen putative inhibiting products (PIPs), selected based on their availability as spontaneous plants or agro-industrial wastes, together with a commercial resistance inducer, were screened in in vitro assays against a collection of 31 phytopathogenic fungi. The inhibitory activity (IA) from the PIPs was evaluated, and statistical analyses were performed to identify the best performer. Several PIPs showed significant inhibitory activity against several fungal species, while others promoted fungal growth, highlighting the dual nature of the tested PIPs as potential bio-fungicides and growth-promoting agents for beneficial fungi. These findings highlight the value of plant-derived metabolites and agricultural waste valorization as promising sources for the development of sustainable botanical fungicides as well as support the transition toward eco-friendly crop protection strategies aligned with the European Green Deal objectives.

Keywords: phytometabolites; bioactive compounds; fungal species; agro-industrial waste

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1. Introduction

Fungal diseases represent one of the most significant problems for agricultural production, causing severe yield losses and reduced crop quality. The management of these diseases has traditionally been performed with chemical fungicides because they are easily accessible, simple to use, and more effective than other alternatives. However, although pesticides are easy to access, their use produces several disadvantages, such as chemical fungicides' persistence in edible plant products, bioaccumulation and biomagnification in soils, and toxicity towards non-target organisms, including humans and animals [1]. In addition, runoff from treated fields can contaminate aquatic ecosystems and natural water sources. Another big disadvantage is the increase in fungicide resistance in fungi, affecting agricultural crops by reducing their efficacy [2]. Furthermore, the long-term exposure to synthetic fungicides has also been associated with the comparison of chronic human diseases and environmental problems such as ozone layer depletion [3]. During these last years, the growth in reporting on studies on phytometabolites (plant extract,

essential oils, gums, and resins) and botanical fungicides has considered them putative goods and sustainable alternatives to chemicals, demonstrating significant antifungal and antimicrobial properties in both in vitro and in vivo trials [3–5]. In fact, the great diversity of plant species offers a rich source of bioactive compounds that can be used as agrochemicals. Cowan [6] estimated that more than 250,000 higher plant species possess bioactive compounds (metabolites) with agrochemical and pharmaceutical applications.

The study and the use of sustainable alternatives to chemicals are, according to the European Green Deal and the EU Farm to Fork Strategy, a main goal with regard to the intention to reduce by 50% the overall use and risk of chemical pesticides by 2030, while promoting the development of safety, sustainability, and nature-based plant protection products.

Among the bioactive compounds, both primary metabolites (proteins, carbohydrates, and fatty acids) and secondary metabolites belong to a phenol class and are of particular interest for the development of botanical fungicides because they are biodegradable, less persistent in the environment, and show different mechanisms of action that minimize the risk of cross-resistance [1]. The secondary metabolites inhibit spores' germination, mycelia growth, germ tubes elongation, and sporulation, as well as interfere with the production of critical enzymes, DNA, and proteins [3]. Additionally, they cause structural alterations in the hypha and mycelia, which prevent the spread of fungi such as *Aspergillus* spp. and *Fusarium* spp. from producing toxic compounds like aflatoxin and fumonisin, respectively [3]. For these reasons, the botanical fungicides can represent healthier, eco-friendlier, and more sustainable alternatives to the chemical fungicides, since they do not degrade soil properties or pose risks to human health, and they minimize the environmental impact and consumer exposure to the danger. In addition, they do not harm beneficial soil microorganisms, preserving soil health, and they exhibit no cross-resistance due to their distinct mechanisms of action compared to synthetic fungicides. In recent years, the valorization of agro-industrial wastes has emerged as a fundamental key of the circular economy, aiming to reduce the waste for the industry and maximize the sustainable re-use of natural sources [7]. Studies on the re-use of residues from essential oil extraction (e.g., bergamot), pomegranate juice extraction, or artichoke harvesting have normally considered agro-industrial wastes, showing that they may contain rich sources of bioactive compounds that can be used in agriculture to control several bacterial and fungal pathogens [8–10].

There are several reports on the antifungal activities of phytochemicals against phytopathogenic fungi [11]. Many plant extracts, from species belonging to *Asteraceae*, *Amaryllidaceae*, *Apocynaceae*, *Boraginaceae*, *Cistiaceae*, *Convolvulaceae*, *Crassulaceae*, *Euphorbiaceae*, *Fabaceae*, *Gramineaceae*, *Labiate*, *Lamiaceae*, *Malvaceae*, *Meliaceae*, *Myrtaceae*, *Nyctaginaceae*, *Orchidaceae*, *Punicaceae*, *Rubiaceae*, *Solanaceae*, *Tiliaceae*, *Verbenaceae*, and *Ziginberaceae* families, have been extensively studied for protecting several fruits and vegetables from diseases, including blueberry dieback (by *Lasiodiplodia pseudotheobromae*) [12], okra seed rot and seedlings death (by *Macrophomina phaseolina*) [13], banana anthracnose (by *Colletotrichum musae*) [14], citrus fruit decay (by *Geotrichum candidum*) [15], black moulds in tomato ripe fruits and blight of pepper crops (by *Fusarium solani*) [16], early and late blight of tomato (by *Alternaria solani*) [17], wilt disease of tomato (by *Fusarium oxysporum* f. sp. *lycopersici*) [18], sugar beet damping-off (by *Agroathelia rolfsii*) [19], white yam anthracnose (by *Colletotrichum gloeosporioides*) [20], tomato damping-off diseases (by *Rhizoctonia solani*) [21], wilt and root rot of tomato (by *Fusarium solani*) [22], late blight disease of potato (by *Phythopthora infestans*) [23], artichoke diseases (by *Fusarium solani* and *Verticillium dahliae*) [24], pepper phytophthora blight (by *Phytophthora capsici*) [25], and mango anthracnose (by *Colletotrichum gloeosporioides*) [26].

Botanical fungicides offer several advantages compared with chemical fungicides, including resistance inhibition, eco-friendliness, effectiveness, and selectivity, frequently

resulting in being more cost-effective and easier to prepare for farmers [27]. Only a small number of them have been approved for commercial use due to the many challenges that restrict their adoption and utilization for a wider scale of production. In addition, farmers' reluctance, the lack of standardized formulation techniques, strict legislation, rapid degradation, and other factors further compromise their adoption and utilization.

Due to all of these reasons, the present work aims to provide a preliminary screening of fifteen putative inhibiting products (PIPs) as botanical fungicides, chosen on the basis of their ready availability as spontaneous plants and as agro-industrial wastes, and a commercial product as a resistance inducer against a pool of 31 fungal pathogens by setting up trials in in vitro conditions.

2. Results

2.1. Inhibitory Activity by Putative Inhibiting Products (PIPs) at 2%

Figure 1 reports an example of the efficacy of seven PIPs (2%; *w*/*v*) on three fungal pathogens used in this study and the results show the most sensitive (*P. clamydospora*), the intermediate sensitive (*C. granati*), and the least sensitive (*C. luteo-olivacea*). Detailed data about the inhibition activity played by any PIP is described in Figure 2.



Figure 1. Inhibitory activity played by *P. granatum*, *C. bergamia*, *S. lycopersicum* (leaf), *A. zerumbet*, *U. dioica* and EP5-product on *P. chlamydospora*, *C. granati*, and *C. luteo-olivacea* proved to be the most sensitive, the medium sensitive, and the least sensitive fungi.

Factorial ANOVA analysis showed F values related to the 31 fungal species of 216.14 [degree freedom (df) = 30; $p < 0.001$], and also those related to PIPs of 2233.00 (df = 17; $p < 0.001$). Moreover, factorial ANOVA demonstrated that significant difference was detected between fungus and PIP with an F value of 53.87 (df = 510; $p < 0.001$).

According to Shapiro–Wilk tests, data from fungal species sensitivity and PIPs efficacy followed normal distributions. The Levene tests revealed that the homogeneity of variance was significant, with F values of 7.27 ($p < 0.001$) and 84.69 ($p < 0.001$), respectively.

In Figure 2a, the results as mean values of minimal squares from one-way ANOVA by Tukey's Test ($p < 0.001$) regarding the ability of 18 PIPs used at 2% (*w*/*v*) to inhibit the mycelia growth of 31 fungal pathogens in in vitro trial performed on PDA medium are shown. The fungal sensitivity patterns might be distinguished in three groups. In the first group, the fungal species more sensitive to inhibitory activity overall played by PIPs are included, such as *P. chlamydospora*, which suffered a significant average of IA of 48.99%. The second group includes *P. ramiseptata*, *F. avenaceum*, *M. fructicola*, *P. melonis*, *P. cucumerina*, and *M. laxa* (ML2), showing moderate inhibition with significant IA percentages ranging

from 30.81% to 25.13%. The third group, comprising *P. scolyti*, *C. acutatum*, *V. dahliae*, *M. laxa*, *C. granati*, *C. fioriniae*, *C. incompta*, *T. angustata*, *M. fructigena*, *P. minimum*, and *S. vitis-vinifera*, displayed light inhibition with IA values ranging from 24.65% to 21.09%. The IA values for the remaining 12 fungal species were not significant as very little inhibited (IAs = from 19.26% to 7.64%), confirming that PIP efficacy is strongly fungus dependent. Finally, *A. rolfsii* was absolutely not inhibited, proving to be completely insensitive.

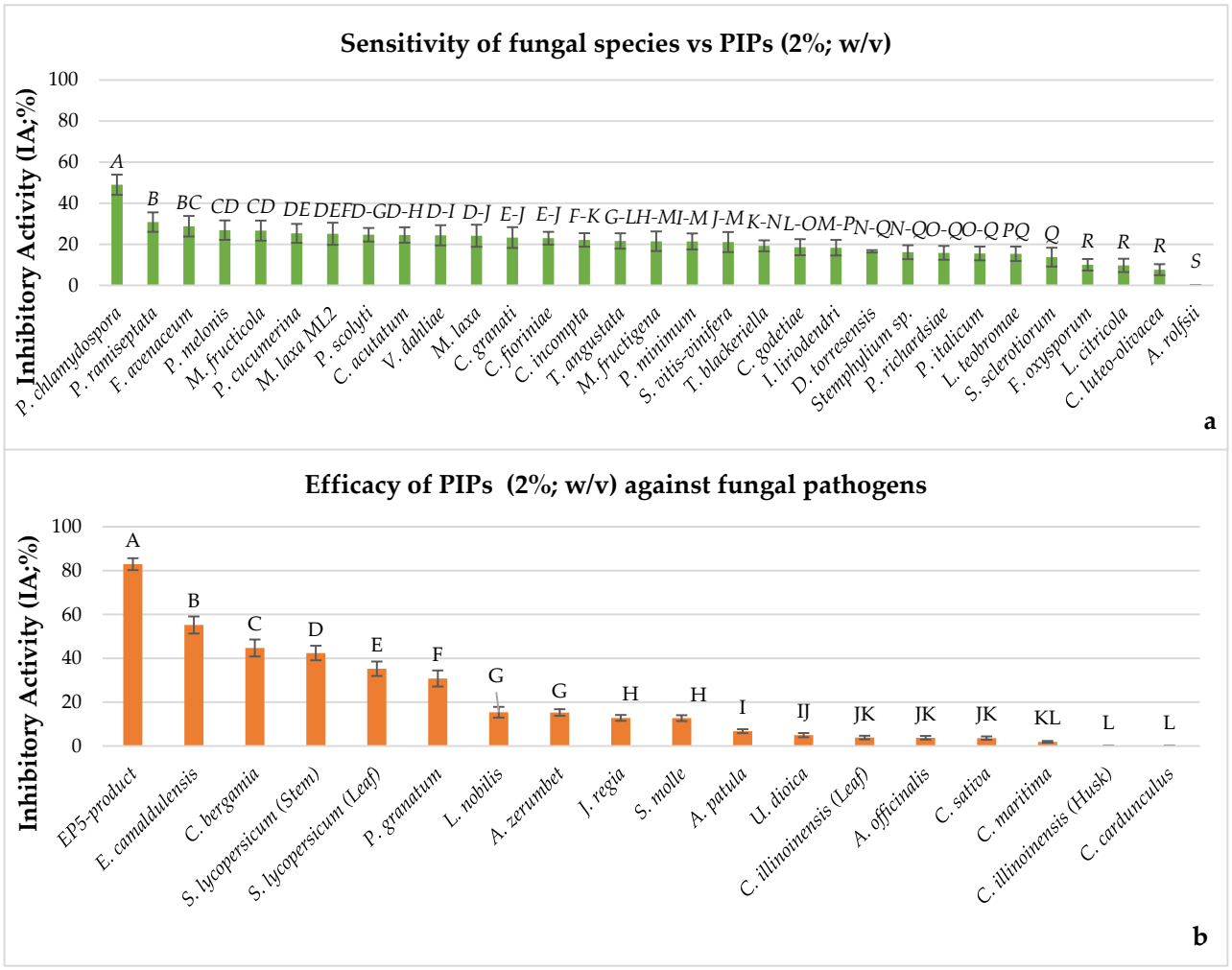


Figure 2. Inhibiting activity played by 18 putative inhibiting products (PIPs), used at 2% (*w*/*v*) under in vitro conditions against 31 fungal pathogens. (**a**) IA mean values representing the sensitivity of fungal species to the effect of PIPs; (**b**) mean values representing the efficacy of all the PIPs used at 2% (*w*/*v*). One-way ANOVA analyses—bars sharing the same letter are not significantly different according to Tukey's HSD test ($p < 0.01$).

In Figure 2b, the results of the efficacy of PIPs to contrast with the overall mycelial growth of the 31 fungal species in Petri dishes are illustrated. The most effective PIP was the EP5-product, with a significant average IA value of 82.90%. In particular, this PIP was able to control the fungal growth of 29 fungal species with an IA from 100% to 73.98%. Only *C. incompta* and *A. rolfsii* showed little or no inhibition (Table S1). *Eucalyptus camaldulensis*, *C. bergamia*, *S. lycopersicum* (both stem and leaf), and *P. granatum* also exhibited significantly strong efficacy to moderate antifungal activities with IA values from 55.18% to 30.79%, suggesting that these PIPs are rich in bioactive phytocompounds capable of inhibiting fungal growth mechanisms. In contrast, *L. nobilis*, *A. zerumbet*, *J. regia*, and *S. molle* were weak PIP inhibitors since they showed low IA values from 15.39% to 12.70%. The remaining PIPs, such as *A. patula*, *U. dioica*, *C. illinoinensis* (leaf), *A. officinalis*, *C. sativa*, and *C. maritima*,

were not significantly effective to contrast fungal growth (IA values from 6.76% to 1.9%). *Carya illinoinensis* (husk) showed the lowest IA values, from 34.74% to 4.76%, being able to weakly control only nine fungal species (Table S2). The only fungus most controlled by this PIP was *P. chlamydospora*, with an IA value of 60.07%. Finally, *C. cardunculus* was absolutely ineffective as it was not able to inhibit all fungal growth (Table S1).

Figure 3 reports the results regarding the interaction between PIPs efficacy and fungal species sensitivity in in vitro conditions. The hierarchical cluster analysis grouped PIPs in seven main clusters according to their IA values across the fungal species targets. Any cluster grouping the PIPs with similar efficacy is represented by the same colour in heatmap; the red colour intensity represents the ability to inhibit the fungal growth and the blue represents the opposite (Figure 3a). In particular, the first group included the EP5-product, which resulted in the most effective PIP; the next four groups included *S. lycopersicum* (leaf and stem), *C. bergamia*, *P. granatum*, and *E. camaldulensis*, showing an IA efficacy from high to good. Four PIPs (*A. zerumbet*, *S. molle*, *J. regia*, and *L. nobilis*) clustered in the sixth group, showing intermediate IA efficacy values. Finally, eight PIPs (*A. patula*, *U. dioica*, *A. officinalis*, *C. sativa*, *C. illinoinensis*, both leaf and husk, *C. cardunculus*, and *C. maritima*) clustered in the last group, showing the lowest IA efficacy. The same groups are also mapped in the constellation diagram reported in Figure 3b.

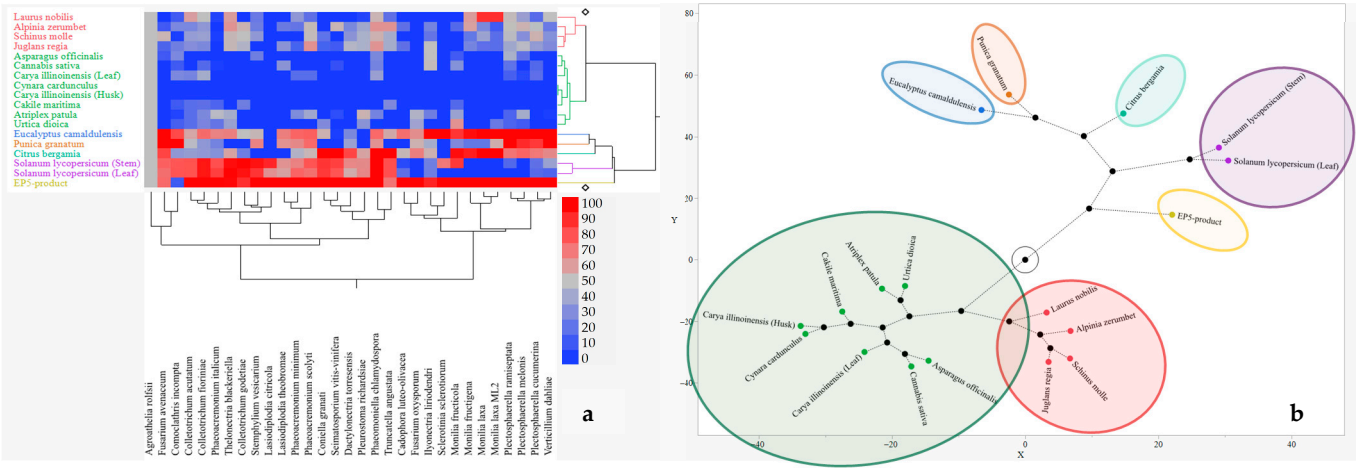


Figure 3. Interaction of PIP efficacy and fungus sensitivity. (**a**) Hierarchal clustering (Ward method) (2%; *w*/*v*) grouping heatmap and seven dendrograms; (**b**) constellation diagram with seven clusters: green circle includes ineffective PIPs; red circle includes the lowest effective PIPs; blue, ochre, light green and purple circles include effective PIPs; and yellow circle also includes the EP5-product as the most effective. Black circles indicate the distance among cluster.

2.2. Fungal Growth Promotion at 2%

Factorial ANOVA analysis showed F values related to the seven fungal species of 202.20 [degree freedom (df) = 6; $p < 0.001$], and also those related to PIPs of 734.70 (df = 17; $p < 0.001$). Moreover, factorial ANOVA demonstrated that significant difference was detected between fungus and PIP, with an F value of 826.39 (df = 102; $p < 0.001$).

According to Shapiro–Wilk tests, data from fungal growth promotion and PIPs efficacy followed normal distributions. The Levene tests revealed that the homogeneity of variance was significant with F value of 5.26 ($p < 0.001$) for fungal species and 13.30 ($p < 0.001$) for PIPs, respectively.

Figure 4 reports data as mean values of minimal squares by one-way ANOVA related to PIP efficacy to promote the Fungal Growth Promotion (FGP) of 7 of the 31 target fungal pathognes. In particular, Figure 4a highlights the ability of all PIPs to promote the fungal growth, although with different intensities. It is possible to divide the PIPs into four groups

based on their ability to promote fungal growth. In details, the first group included *C. illinoinensis* husk and *C. carduculus*, since they were significantly more effective in promoting fungal growth, on average at 104.03%. The second group included *C. maritima*, *A. officinalis*, *C. illinoinensis* (leaf), *C. sativa*, *U. dioica*, and *A. patula*, which were able to promote fungal growth, with values from 101.20% to 94.82%. The third group included *S. molle*, *J. regia*, *L. nobilis*, and *A. zerumbet*, which were able to promote fungal growth with values from 88.32% to 85.13%. The last group included all the PIPs that slightly promoted the fungal growth of *P. granatum*, *S. lycopersicum* (leaf and stem), *C. bergamia*, *E. camaldulensis*, and EP5-product from 71.16% to 17.07%. Figure 4b indicates the fungal species more susceptible to biostimulation activity by PIPS, such as *C. luteo-olivacea* (119.47%), *P. richardsiae* (88.37%), *P. italicum* (87.84%), *S. vitis-vinifera* (87.31%), *C. incompta* and *V. dahliae* (79.95%), and *P. chlamydospora* (53.55%). The same results are shown as single interactions among PIPs and fungal pathogens in Table S3. In particular, *C. illinoinensis* promoted the fungal growth of *C. luteo-olivacea* (FGP = 147.16%), *C. incompta* (FGP = 116.22%), *S. vitis-vinifera* (FGP = 116.24%), *P. chlamydospora* (FGP = 114.54%), *V. dahliae* (FGP = 111.51%), *P. italicum* (FGP = 110.04%), and *P. richardsiae* (FGP = 109.32%). On the other hand, EP5-product did not promote the fungal growth of *P. richardisiae*, *P. chlamydospora*, *S. vitis-vivnifera*, and *V. dahliae* (FGP = 0.00%).

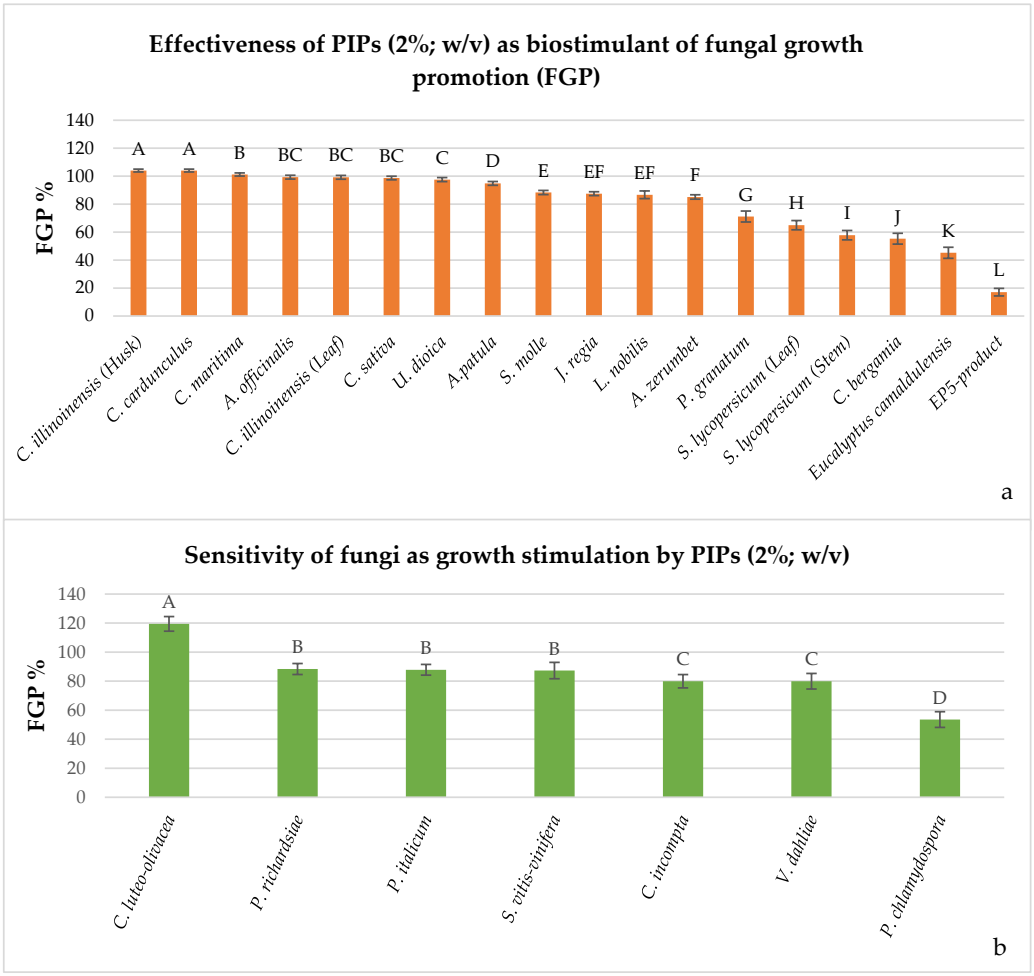


Figure 4. Effectiveness of PIPs in promoting the fungal pathogens' growth in in vitro conditions. (**a**) PIPs with ability to promote fungal growth; (**b**) sensitivity of fungi to the biostimulant activity by PIPs at 2% (*w*/*v*) in in vitro conditions; one-way ANOVA, bars sharing the same letter are not significantly different according to Tukey's HSD test ($p < 0.01$).

Figure 5 graphically illustrates the interaction between PIPs and fungi, regarding the ability of the PIPs to promote fungal growth in plates. Figure 5a shows a hierarchical clustering analysis visualized as heatmap and dendrogram grouping of PIPs in four main clusters based on their growth-promoting activity patterns on fungi: the red colour intensity represents the ability of the PIPs to promote the fungal growth and the blue one a reducted or no ability. The constellation diagram (Figure 5b), by a synthetic visual representation of the relationships and distances among the PIP clusters, highlights those more effective in red and less effective in yellow.

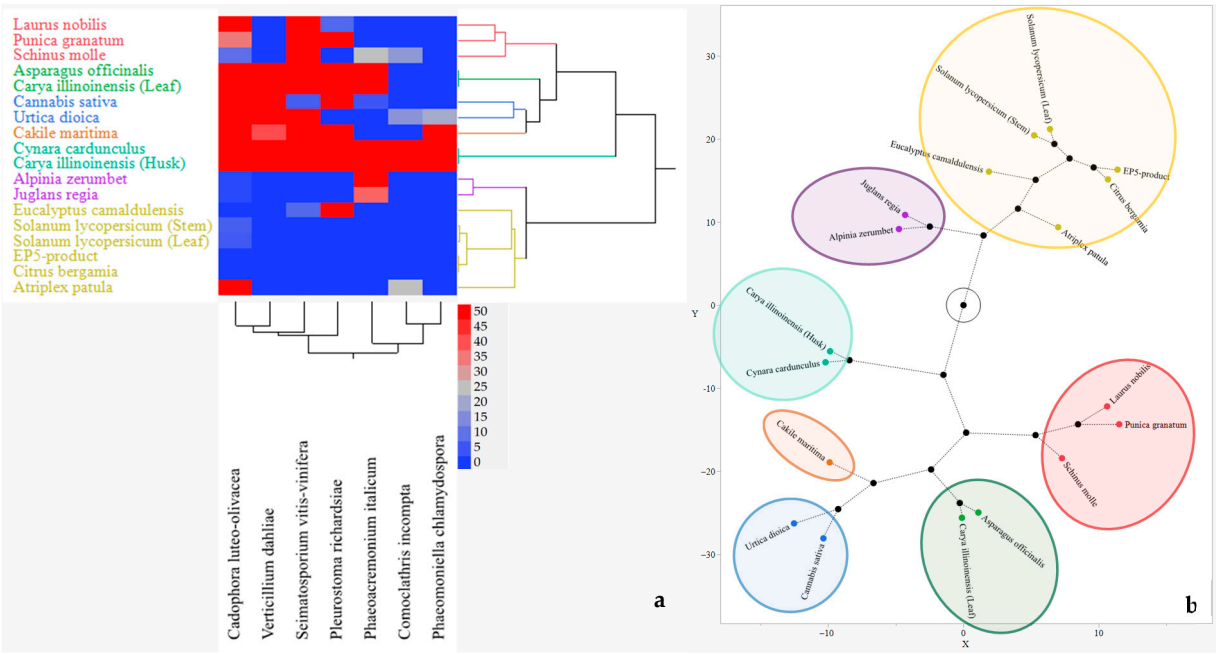


Figure 5. Interaction of PIP (2%, *w*/*v*) efficacy on fungal growth promotion. (**a**) Hierarchical clustering (Ward method); (**b**) constellation diagram with seven clusters: red, green, blue, ochre, and light green circles include the most effective PIPs; purple circle includes effective PIPs; and yellow circle includes ineffective PIPs. Black circles indicate the distance among clusters.

2.3. Inhibitory Activity by Putative Inhibiting Products (PIPs) at 4%

Factorial ANOVA analysis showed F values related to the 31 fungal species of 819.70 [degree freedom (df) = 30; $p < 0.001$], and also those related to PIPs of 8079.61 (df = 5; $p < 0.001$). Moreover, factorial ANOVA demonstrated that significant difference was detected between fungus and PIP with an F value of 322.09 (df = 150; $p < 0.001$).

According to Shapiro–Wilk tests, data from fungal growth promotion and PIPs efficacy followed normal distributions. The Levene tests revealed that the homogeneity of variance was significant with F value of 7.34 ($p < 0.001$) for fungal species and 35.57 ($p < 0.001$) for PIPs, respectively.

Figure 6 illustrates IA values of six PIPs selected for experiment at 4% concentration in order to observe putative increasing effect on their antimicrobial activity. Figure 6a confirms the trend observed from Figure 1 due to dose increasing, a higher fungal sensitivity to the PIPs was recorded. Overall, *P. chlamydospora* remained the most inhibited fungal species with an IA of 80.90%. Conversely, *L. citricola* was the least inhibited fungal species, which was controlled at 0.00%. In particular, the data shown in Figure 6b indicate that, when increasing the concentration at 4% of PIPs, the IA increases favourably with respect to 2% concentration. EP5-product still remains the most effective, reaching 92.14% of IA at 4% concentration, followed by *E. camaldulensis* (57.67%), *C. bergamia* and *P. granatum* (47.64% and 47.45%), *S. lycopersicum* (32.06%), and *S. molle* (18.16%). The difference in increasing

doses (Δ = 4–2%) is particularly evident for *P. granatum*, EP5-product, and *S. molle*, with differential increments of 16.66%, 9.22%, and 5.46%, respectively (Figure 6c). Meanwhile, the Δ was moderately significant for *E. camaldulensis* and *C. bergamia* (2.94%), and not quite significant for *S. lycopersicum* (leaf), which worsened its performance, with a −3.17%. These findings encounter confirmation and are validated by hierarchical clusterization in two ways and by a synthetic visual constellation graph in Figure 7a,b. In particular, in Figure 7a, it is possible to observe the efficacy of the PIPs at 4% concentration, which can be divided into three groups: in the first falls EP5-product; in the second, *E. camaldulensis* and *P. granatum*; and in the third *C. bergamia*, *S. lycopersicum* (leaf), and *S. molle*. The three groups are represented with different colours; the same are present in the constellation diagram.

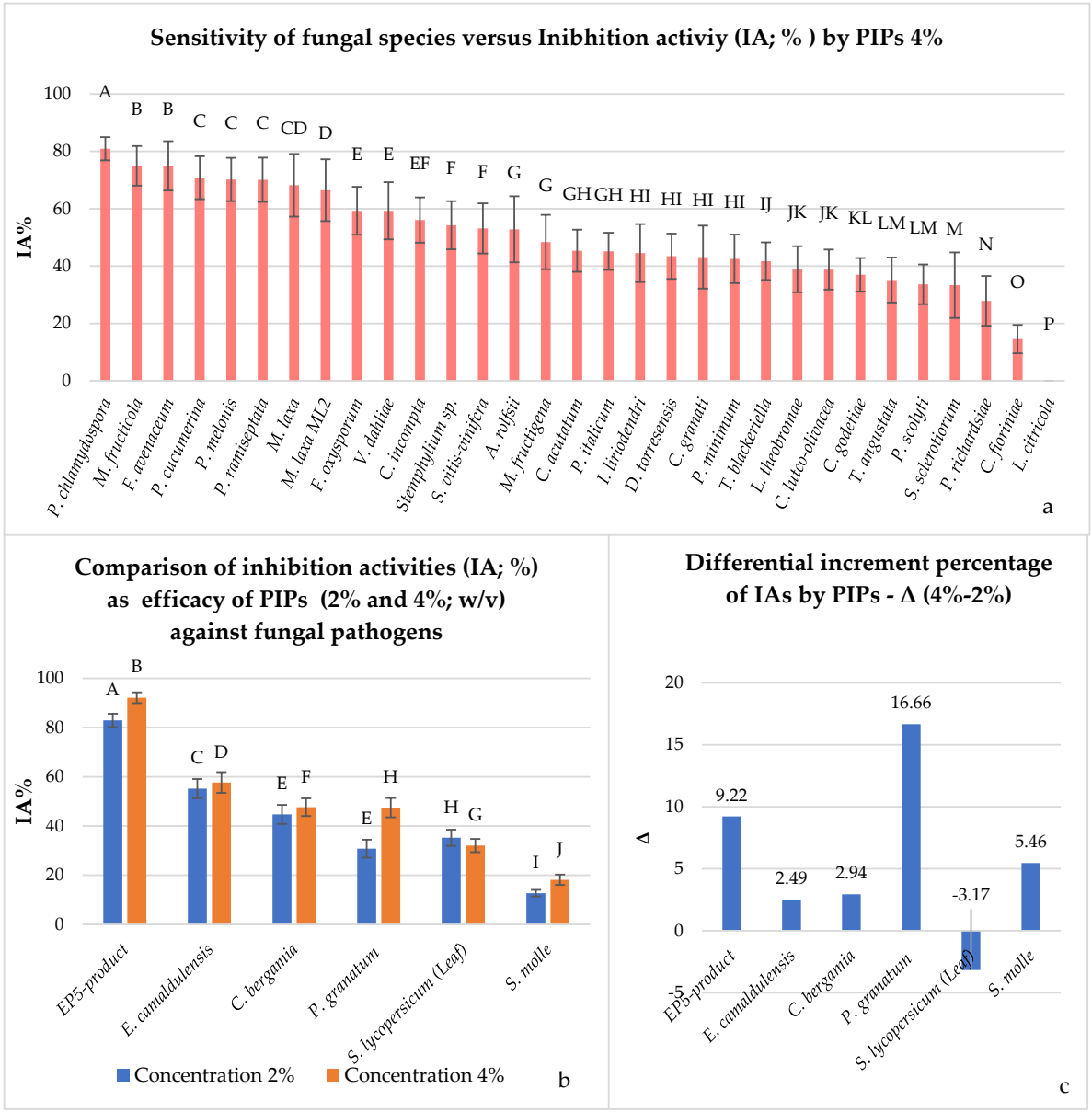


Figure 6. (**a**) Sensitivity expressed from fungal species versus six putative inhibiting products (PIPs) at 4% concentration under in vitro conditions; (**b**) comparison of IA values obtained by PIPs at 2 and 4%; one-way ANOVA analyses, Tukey's test, bars sharing the same letter are not significantly different according to Tukey's HSD test ($p < 0.01$); (**c**) differential increment percentage (Δ = 4–2%) of inhibiting activities (IA) by PIPs.

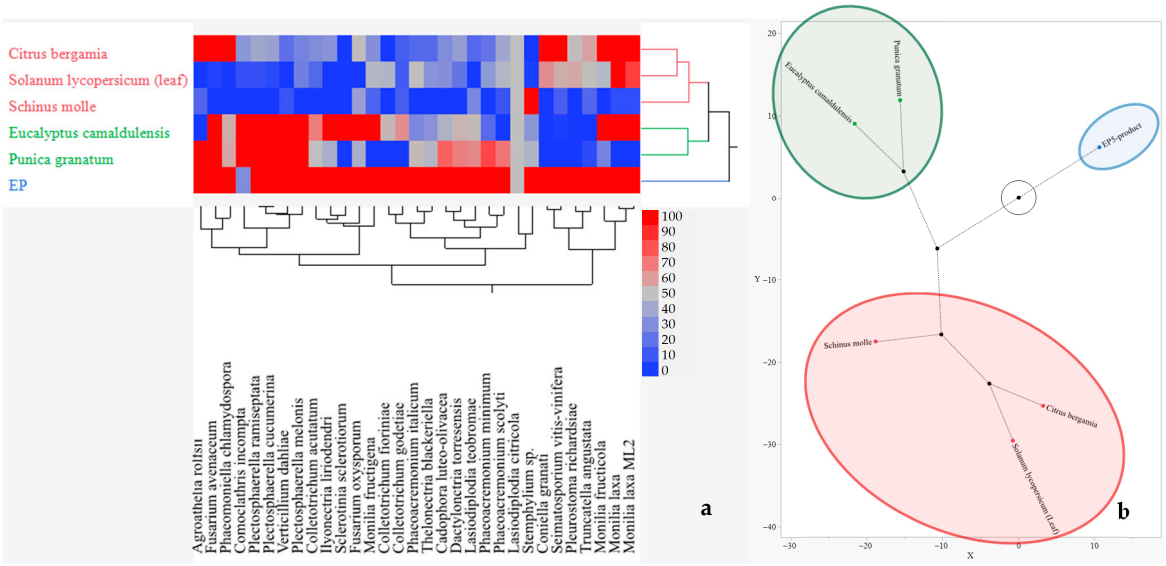


Figure 7. Interaction of PIP efficacy (4%; *w*/*v*) and fungus sensitivity. (**a**) Hierarchical clustering in two ways showing three main PIP clusters by heatmap and dendrograms; (**b**) constellation diagram with three clusters: red circle includes the least effective PIPs; green circle includes effective PIPs; and blue circle includes just EP5-product as the most effective PIP. Black circles indicate the distance among clusters.

Table S2 reports the single interactions among fungal pathogens and PIPs, where the effectiveness of EP5-product, as a reference, increased IA at 100%, going from seventeen fungal species at 2% to twenty-five at 4% of concentration. The same results were obtained also for *E. camaldulensis*, because the numer of fungi inhibited increased from seven (at 2% concentration) to thirteen (at 4% concentration). In addition, when the concentration of *C. bergamia*, *P. granatum*, and EP5-product powders were increased from 2% to 4%, the fungal species *A. rolfsii*, at 2%, was never inhibited, resultin in being totally controlled.

3. Discussion

The results presented provide a comprehensive overview of the biological activities of eighteen putative inhibiting products (PIPs) used as powders, highlighting their dual behaviour as both antifungal inhibitors and growth promoters. The preliminary screening carried out with eighteen PIPs at 2% concentration demonstrated that they were able to exhibit clear differentiation between highly sensitive and resistant fungal species. Generally, both kinds of putative botanical fungicides, spontaneous plants, and agro-industrial wastes were able to produce inhibition activity with different degrees. The hierarchical cluster analysis grouped the tested PIPs into seven main clusters according to their inhibitory activity across the 31 fungal species, providing a first attempt to classify the active plants as bio-fungicides as well as a useful framework for identifying the effective PIPs. Strong inhibitory effects were obtained by EP5-product and some other plant-derived PIPs, such as *E. camaldulensis*, *C. bergamia*, *S. lycopersicum*, and *P. granatum.* EP5-product was the best performing PIP, controlling 29 fungal species with the major IA values. The only two fungal species not inhibited by EP5-product were *A. rolfsii* and *C. incompta*. Carlucci et al. [28] also reported the IA of EP5-product against *B. cinerea* and *M. laxa* in postharvest conditions. The putative efficacy of this PIP is mainly due to the presence of minerals, silicates, and citric acids that create unfavourable fungal growth conditions. Further studies conducted in in vitro and in vivo conditions are needed to confirm these data.

Among the plant-based PIPs, *E. camaldulensis*, *C. bergamia*, *S. lycopersicum*, and *P. granatum* were the best performing, as they controlled more than 68% of fungal species tested. Ameziane et al. [15] tested powders, extract oil, and solvent extracts of 21 PIPs to control postharvest citrus disease caused by *Geotrichum candidum* and *Penicillium* spp., showing that powder by *Eucalyptus globulus* totally inhibited the mycelial growth of both fungi. In addition, Gakuubi et al. [29] showed that essential oil of *E. camaldulensis* had important antifungal activity against five *Fusarium* species, including *F. oxysporum*, *F. proliferatum*, *F. solani*, *F. subglutinans*, and *F. verticillioides*. Similar results were obtained by Salem et al. [30] when essential oil of *E. camaldulensis* was used to control *Alternaria alternata* and *F. subglutinans* species. These authors attributed the putative antifungal activity of *E. camaldulensis* essential oil to the presence of terpenes such as eucalyptol and α-pinene [30]. In a study regarding the potential antifungal activity of extracted oil of *E. camaldulensis* leaves and *Citrus* spp. peel against *Aspergillus* spp. and *F. culmorum*, Elgat et al. [31] confirmed the inhibitory activity due to the presence of β-pinene, linalool, and α-terpineol. Ramaiah and Garampalli [32] showed the IA of water extracts of *E. globulus* and *Solanum indicum* against *F. oxysporum* f. sp. *lycopersici*. Kobayashi et al. [33] demonstrated the antifungal activities of tomato leaf volatile compounds against *F. oxysporum*, f. sp. *Melonis*, and *Colletotrichum gloeosporioides* due to the presence of sesquiterpens (β-caryophillene) and alchols (geraniol and α-linalool). Our results agree with these studies since *E. camaldulensis*, *C. bergamia*, and *S. lycopersicum* played an IA on *F. avenaceum* and *P. chlamydospora* higher than 80.00%. Meanwhile, *F. oxysporum* was controlled by *E. camaldulensis* and *C. bergamia* (IA values higher than 50.00%) but not by *S. lycopersicum* (IA value 0.00%). Moreover, Dias et al. [34] demonstrated the IA of essential oils from the fruit peel of three *Citrus* species against *S. sclerotiorum* in in vitro conditions, while Olakunle et al. [35] showed IA of ethanolic extract of *C. sinensis* on *Aspergillus niger* and *A. alternaria*. Conversely, our results showed that *C. bergamia* powder was unable to inhibit *A. rolfsii*, *Lasiodiplodia* species, and *S. sclerotiorum*. Moreover, low IA values (<50%) were obtained against *Colletotrichum* and *Phaeoacremonium* species, *C. granati*, *I. liriodendri*, *S. vesicarium*, and *T. blackeriella*.

Li Destri Nicosia et al. [36] and El Khetabi et al. [37] reported the IA of phenolic compounds from water and ethanolic extracts of *P. granatum* peel against postharvest pathogens such as *Botrytis cinerea*, *Monilia*, and *Penicillium* species. Our results showed low IA (<50%) of *P. granatum* powder on *C. granati*, *S. sclerotiorum*, *Colletotrichum*, and *Monilia* species, and high IA (>80%) against *F. avenaceum*, *C. incompta*, *Plectosphaerella* species, *P. chlamydospore*, and *V. dahliae.*

The hierarchical clusterization grouped seven powder plants as the worst PIPs. They showed IA values < 40%, except for *A. officinalis*, *C. sativa*, and *C. illinoinensis* (leaf), which showed higher IA values only *against P. chlamydospora*.

Conversely, the results observed across the analyses carried out in this study showed that all PIPs, especially *A. officinalis*, *A. patula*, *C. sativa*, *C. maritima*, *C. illinoinensis* (leaf and husk), *C. cardunculus*, and *U. dioica*, promoted fungal growth of 7 out of the 31 fungal species tested (*C. luteo-olivacea*, *P. italicum*, *C. incompta*, *P. richardsiae*, *P. chlamydospora*, *S. vitis-vinifera*, and *V. dahliae*), exhibiting significant effects likely due to their nutritional or signalling properties. In particular, *C. illinoinensis* husk and *C. cardunculus* powders failed to control any fungi since promoting their growth, while EP5-product and *E. camaldulensis*, as strong inhibitors, were able to exhibit minimal or no stimulation on fungal growth, probably due to their metabolomic activities which depend on the biological nature. The hypothesis is that certain plant materials are rich in carbohydrates (complexed and simple), lipids, and proteins and poor of antimicrobial compounds such as polyphenols, terpen, and sterols. Uppala et al. [38] reported that plant-based culture media supplemented with dried parts of rise, sorgum, and barnyard grass plants improved the growth and

sporulation of fungal species *Cercospora janseana*, while Osman et al. [39] showed the efficacy of plant materials such as fresh vegetables (tomato, aubergine), legumes (pigeon pea), and seeds (wheat and dukhum) to promote fungal growth of *Aspergillus* species, *Curvularia lunata*, and *F. oxysporum.* The putative inefficacy of the powders mentioned above disagrees with many studies that reported their IA when extracted with organic solvents. In particular, Osorio et al. [40] asserted that methanol 70% extracts of *C. illinoinensis* husk had a fungicide effect against *Phytium* sp., *Colletotrichum coccodes*, *C. truncatum*, *F. sambucinum*, and *Rhizoctonia solani* and a fungistatic effect against *F. solani*, *F. verticillioides*, and *A. alternata*. This efficacy is attributable to the presence of a high concentration of extracted polyphenols and tannins [41]. Moreover, Barbosa et al. [42] reported the antifungal activity of methanolic and ethanolic extracts from *C. cardunculus* against *Aspergillus* spp. due to the presence of chlorogenic acid, apigenin, and luteolin. Methanol extract of *U. dioica* had strong antifungal activity against *A. alternata* because of the presence of phenolic acids (ferulic and p-coumaric) [43]. The water extract of *A. officinalis* contained flavonoids that had great IA on *F. oxysporum* [5]. Skadhauge et al. [44] also report that flavonoids, such as proanthocianiodins and dihidroquercitins, are involved in defence mechanisms of barley against *Fusarium* species. All these examples encourage a further investigation of the potential antifungal properties of the fungal growth-promoting plant powders tested in this study by extracting them with organic solvents or as potential bio-stimulant uses for beneficial microorganisms in rhizosphere [45].

The results obtained when increasing the concentration of certain PIPs to 4% allowed us to observe that the increased efficacy of antifungal activity is directly associated with some PIPs but not for all. In particular, when the concentration of *C. bergamia*, *P. granatum*, and EP5-product powders were increased from 2% to 4%, the fungal species *A. rolfsii*, that at 2% was never inhibited, became totally controlled. These conflicting activities lead to well-evaluated concentrations, fungal physiology, and plant metabolites in determining biological outcomes. Therefore, target-specific screening and concentration optimization are relevant to ensure that PIPs are used appropriately for either inhibitory or stimulatory purposes. Moreover, these results suggest that the inhibiting activities by PIPs against fungi is dose-dependent since the toxicity increased as PIPs concentration increased [19], although more studies are needed to evaluate putative phytotoxic effects due to higher doses. The variability of differential increment among the six PIPs implies that they exhibit variable capacity, because some PIPs might be more efficient at lower concentrations and others need higher doses to reach comparable inhibition.

These results demonstrated that PIPs do not act uniformly against all fungal taxa but possess distinct antifungal activities, probably influenced by plant metabolite composition, structure, and mechanism of action. In contrast, the lack of inhibition towards certain fungi, such as *A. rolfsii*, indicates that PIPs antifungal mechanisms may rely on specific molecular targets not present in resistant fungal species. This specificity suggests that PIPs can operate through targeted biochemical interactions which could reduce off-target effects and environmental risks compared to broad-spectrum synthetic fungicides.

Anyway, this preliminary study is valuable for further investigations regarding identifying high-performing PIPs and guiding future mechanistic or formulation studies aimed at exploiting their potential in agriculture or biotechnology. Moreover, this study provides important findings for sustainable applications of PIPs able to exhibit multifunctional and context-dependent activities. Their dual nature might lead to both opportunities and risks, because PIPs with strong antifungal activity represent promising candidates as botanical fungicides, while those with growth-promoting properties may be useful in non-pathogenic fungal enhancement (as mycorrhizal production and/or composting processes) [45], but at

the same time, it requires careful evaluation before field applications, as some PIPs could enhance severe fungal pathogen proliferation.

Overall, these findings recognize the strong potential of PIPs as innovative tools in sustainable plant protection while emphasizing the need for targeted application strategies to maximize their efficacy and minimize their detrimental and undesirable effects. In conclusion, these results indicate that tested PIPs could serve as promising biocontrol candidates or natural antifungal agents in sustainable agriculture. The large number of severe fungal pathogens for several agricultural crops and plant products as putative botanical fungicides used in this preliminary study offers important ideas for further investigations in crop protection, postharvest preservation, and formulation of eco-friendly antifungal products, although some negative aspects deserve more attention, such as phytotoxicity, cost, and environmental persistence in follow-up in vitro studies. Further studies will be carried out in order to test the more effective PIPs in greenhouse trials and in the open field. Other studies are necessary to ascertain the composition and toxicity of metabolites of plants like promising botanical fungicides, as their chemical compounds could be dangerous for humans.

4. Materials and Methods

4.1. Collecting and Preparation of Plant Powders as Putative Botanical Fungicides

Various plant organs (leaves, stems, fruit husk, and peel) from fifteen plant species, such as spontaneous, ornamental plants, and agro-industrial wastes, supposed to have potential antifungal activity (PIP; putative inhibiting products), were collected in the Apulia region (Southern Italy). The botanical species, the kind of plant organs, and the collecting areas are reported in detail in Table 1. Of the fourteen plant species, two of them (*Carya illinoinensis* and *Solanum lycopersicum*) were selected for two different kinds of plant organs, such as leaves and husk and leaves and stems, respectively, for a total of 18 PIPs (Table 1). The plant tissues were sectioned in small portions and left to dry in room conditions. After the fresh weight was reduced by at least 30–40%, the plant tissues were moved into the stove at 30 °C (FN 500 Sterilizer, Nuve, Ankara, Turkey). When they reached the constant weight, the dried plant tissues were ground as finely as possible using an electric mill equipped with a fine-mesh screen, 2 mm of diameter, (Geomill-50P, Geo Tech, Perugia, Italy). The resulting powders were stored in labelled airtight containers. In addition, a commercial product (EP5-Protect) as a resistance inducer for organic agriculture was included in in vitro assays. Its composition consists of minerals and silicates such as bentonite (30%), citric acid (5%), iron (10%), zinc (6%), manganese (6.5%), and sulphate (Phenom Biotech srl, Milan, Italy).

Table 1. Detailed information of the fifteen botanical species tested in this study.

Plant Species	Botanical Family	Common Name	Kind and/or Use	Locality	Plant Organ
Alpinia zerumbet B.L. Burtt e R.M. Sm.	*Zingiberaceae*	Alpinia	Ornamental plant	Italy, Apulia, Cerignola	Leaf
Asparagus officinalis L.	*Asparagaceae*	Asparagus	Agro-industrial waste	Italy, Apulia, Manfredonia	Turion
Atriplex patula L.	*Amaranthaceae*	Atriplex	Spontaneous plant	Italy, Apulia, Margherita di Savoia	Leaf
Cakile maritima (L.) Scop.	*Brassicaceae*	European searocket	Spontaneous plant	Italy, Apulia, Margherita di Savoia	Leaf

Table 1. *Cont.*

Plant Species	Botanical Family	Common Name	Kind and/or Use	Locality	Plant Organ
Cannabis sativa L.	*Cannabaceae*	Hemp	Agro-industrial waste	Italy, Apulia, Lucera	Leaf
Carya illinoinensis (Wangenh.) K. Koch.	*Juglandaceae*	Pecan	Agro-industrial waste	Italy, Apulia, Cerignola	Husk, Leaf
Citrus bergamia Risso & Poit.	*Rutaceae*	Bergamot	Agro-industrial waste	Italy, Calabria, Reggio Calabria	Peel
Cynara cardunculus L.	*Asteraceae*	Artichoke	Agro-industrial waste	Italy, Apulia, Orta Nova	Leaf
Eucalyptus camaldulensis Dehnh.	*Myrtaceae*	Red river gum	Agro-industrial waste	Italy, Apulia, Foggia	Leaf
Juglans regia L.	*Juglandaceae*	Walnut	Agro-industrial waste	Italy, Apulia, Cerignola	Leaf
Laurus nobilis L.	*Lauraceae*	Bay laurel	Agro-industrial waste	Italy, Apulia, Stornarella	Leaf
Punica granatum L.	*Punicaceae*	Pomegranate	Agro-industrial waste	Italy, Apulia, Foggia	Peel
Schinus molle L.	*Anacardiaceae*	False pepper	Ornamental plant	Italy, Apulia, Manfredonia	Leaf
Solanum lycopersicum L.	*Solanaceae*	Tomato	Agro-industrial waste	Italy, Apulia, Orta Nova	Leaf, Stem
Urtica dioica L.	*Urticaceae*	Nettle	Spontaneous plant	Italy, Apulia, Orta Nova	Leaf

4.2. Fungal Pool-Target Pathogens

The antifungal activity of PIPs was proved against thirty-one fungal pathogens from the private collection of the 'Plant Pathology and Diagnosis' laboratory in the Department of Sciences Agriculture, Food, Natural Resources and Engineering at the University of Foggia, Italy. The fungal pool-target pathogens included soil-borne, wood-decay, and fruit rot fungi (Table 2). All fungal pathogens have been isolated from a wide range of high-value horticultural and fruit crops cultivated mainly in the province of Foggia (Apulia region). Their taxonomic identification has been provided by molecular tools as reported in Table 2. Briefly, DNA was extracted according to Carlucci et al. [46]; based on the fungal genera, different target genes were amplified (Table 2). The resulting amplicons were subsequently sequenced; the consensus sequences were compared with reference strains (ex-type) available in the GenBank database, using the Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov (accessed on 10 April 2025).

Table 2. Fungal phytopathogens used to assess the inhibitory activity (IA) of the selected putative inhibiting products (PIPs).

Fungal Species	Disease/Pathogen Attitude [a]	Host Plant	Locality	Gene Used for Taxonomic Identification [b]
Agroathelia rolfsii	Southern blight (SBP)	Tomato	Italy, Apulia, Torremaggiore	ITS
Cadophora luteo-olivacea	TD; WDP	Grapevine	Italy, Apulia, Torremaggiore	ITS, *tef 1-α* and *β-tub*
Colletotrichum acutatum	Anthracnose (FRP)	Olive tree	Italy, Apulia, Turisiano	ITS, *β-tub*, *act*, *gapdh* and *chs-1*;

Table 2. *Cont.*

Fungal Species	Disease/Pathogen Attitude [a]	Host Plant	Locality	Gene Used for Taxonomic Identification [b]
C. fioriniae		Grapevine	Italy, Apulia, Bari	
C. godetiae		Olive tree	Italy, Apulia, Casarano	
Comoclathris incompta	Shoot necrosis disease (SBP)	Cabbage	/	ITS
Coniella granati	Pomegranate dieback (WDP)	Pomegranate tree	Italy, Apulia, L. of Emmaus	ITS
Dactylonectria torresensis	Black foot disease (SBP)	Grapevine	Italy, Apulia, Foggia	ITS, *β-tub* and *his-3*
Fusarium avenaceum	Fusarium head blight (SBP)	Wheat	Italy, Apulia, Foggia	ITS and *tef 1-α*
Fusarium oxysporum	Vascular wilt (SBP)	Onion	Italy, Apulia, Margherita di Savoia	ITS and *tef 1-α*
Ilyonectria liriodendri	Black foot disease (SBP)	Grapevine	Italy, Apulia, Cerignola	ITS, *β-tub* and *his-3*
Lasiodiplodia citricola	Botryosphaeria dieback (WDP)	Pomegranate tree	Italy, Apulia, Foggia	ITS and *tef 1-α*
L. theobromae				
Monilia fructicola	Brown rot (FRP)	Peach tree	Italy, Apulia, Bari	ITS and *tef 1-α*
M. fructigena		Quince	Italy, Apulia, Foggia	
M. laxa		Apricot	Italy, Apulia, Canosa di Puglia	
M. laxa ML2		Apricot	Italy, Apulia, Bari	
Phaeoacremonium italicum	TD; WDP	Grapevine	Italy, Apulia, Foggia	*β-tub* and *act*
P. minimum		Grapevine	Italy, Apulia, Foggia	
P. scolyti		Olive tree	Italy, Apulia, Monte s. Angelo	
Phaeomoniella chlamydospora	TD; WDP	Grapevine	Italy, Apulia, San Severo	ITS and *β-tub*
Plectosphaerella ramiseptata	Vascular rot (SBP)	Cauliflower	Italy, Foggia, Borgo Cervaro	ITS, *tef 1-α* and *rpb2*
P. cucumerina		Melon	Italy, Apulia, Foggia	
P. melonis		Watermelon	Almenara (ES)	
Pleurostoma richardsiae	TD; WDP	Olive tree	Italy, Apulia, Canosa di Puglia	ITS
Sclerotinia sclerotiorum	Sclerotinia stem rot (SBP)	Broccoli	Italy, Apulia, Foggia	ITS
Seimatosporium vitis-vinifera	TD; WDP	Grapevine	Italy, Apulia, Canosa di Puglia	LSU, ITS, *tef 1-α* *β-tub* and *rpb2*
Stemphyliumvesicarium	Stemphylium leaf spot (FRP)	Cabbage plant	Italy, Apulia, Foggia	ITS and *gapdh*
Thelonectria blackeriella	Black foot desease (SBP)	Grapevine	Italy, Apulia, San Severo	LSU, ITS, *β-tub*, *act* and *rpb1*
Truncatella angustata	TD; WDP	Grapevine	Italy, Apulia, Altamura	LSU, ITS, *tef 1-α*, *β-tub* and *rpb2*
Verticillium dahliae	Verticillium wilt (SBP)	Olive tree	Italy, Apulia, Canosa di Puglia	ITS and *β-tub*

[a] = SBP = soilborne pathogen; FRP = fruit rot pathogen; WDP = wood decay pathogen; TD = trunk disease. [b] = ITS = internal transcribed spacer; *β-tub* = beta tubulin gene; *act* = actin gene; *gapdh* = glyceraldehyde-3-phosphate dehydrogenase; *chs-1* = chitin synthase; *his-3* = histone; *tef 1-α* = elongation; factor; *rpb2* = second-largest subunit of DNA-directed RNA polymerase II; LSU = large subunits; *rpb1* = RNA polymerase II subunit 1.

4.3. Inhibitory Activity Against Fungal Pathogens by Putative Inhibiting Products (PIPs)

The 2% (*w*:*v*) of each plant powder (from 15 different botanical taxa) and the EP5-product were supplemented with potato dextrose agar media (PDA; 39 g l-1; Sigma-Aldrich, Milan, Italy) and then thermally treated at 115 °C for 15 min in autoclave. For citrus bergamia and EP5-product, before the thermal treatment, the pH was adjusted to 6.0 with NaOH 3M. Fungal plugs of 0.4 mm in diameter of each 31 fungal pathogens were placed at the centre of Petri dishes of 90 mm containing the PDA medium added with PIPs and incubated in darkness at 21 ± 3 °C up to 21 days. The same fungal species inoculated on PDA media without PIPs were used as the control. The experiment was replicated three times. The mycelial growth was detected every 48 h, taking perpendicular diameter values up to 21 days. The inhibitory activity (IA; %) was calculated as the percentage of mycelium growth inhibition compared to the control by the following formula:

$$IA = \left(\frac{D_1 - D_2}{D_1} \right) \times 100$$

where D_1 was the measurement of the perpendicular diameter of the fungal colony on PDA control plates, and D_2 was the measurement of the perpendicular diameter of the fungal colony on PDA plates added with PIPs. To tentatively increase the inhibiting activity of some of the PIPs, bergamot, eucalyptus, false pepper, pomegranate, tomato, and EP5-product were tested at 4% of plant powders against the same fungal pathogens.

4.4. Statistical Analyses

The statistical analyses were performed by JMP software package, version 16.2 (SAS Institute Inc., Cary, NC, USA) on the datasets collected during both experiments (2% and 4% PIPs). The percentage data of inhibition activity (IA) were arcsine root-square transformed in Excel 2007 by the formula DEGREES(ASIN(SQRT(X))), where X is the percentage value.

To test if the datasets followed normal distributions, the Shapiro–Wilk test (W test) was used. Homogeneity of variance of datasets on the base of 31 fungal species and of 18 PIPs was assessed using the Levene test. A factorial ANOVA analysis was performed to determine the significance in the difference of IA by PIPs against fungal growth, the differences due to sensitivity of each fungus assayed, and to detect any interaction between these factors (Fungus × PIPs). One-way ANOVA was performed to determine the significant differences in inhibiting activity (IA) caused by each fungal species tested, and any differences due to the eighteen PIPs used. Tukey's tests were used for the comparison of the treatment means, at $p < 0.01$.

A hierarchical clustering analysis by the Ward method and constellation diagram were carried out by JMP software package, version 16.2 (SAS Institute Inc., Cary, NC, USA) to show the interaction and the behaviour of fungal species and PIPs tested.

The same statistical analyses were also performed for the dataset obtained from fungal growth promotion.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants14233634/s1, Table S1: Interactions between fungal pathogens and PIPs relating to IA (2%; *w*/*v*); Table S2: Interactions between fungal pathogens and PIPs relating to IA (4%; *w*/*v*); Table S3: Interactions between fungal pathogens and PIPs relating to fungal growth promotion (FGP).

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Objective 2. Evaluation the *in vitro* antifungal potential of seven PIPs against ten soilborne fungal pathogens, and assessment their potential application for biofumigation and as biofungicides *in vivo* against three specific soilborne fungal pathogens of wheat.

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Efficacy of Putative Botanical Fumigants and Fungicides Against Soil-Borne Fungal Pathogens in Wheat

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ABSTRACT

The negative effects of pesticides for humans and environment have encouraged research into natural control means to protect crops from biotic stresses. This study evaluated the antifungal activity and phytotoxicity of seven natural putative inhibitory products (PIPs), as well their persistence, under both *in vitro* and *in vivo* conditions. Six agricultural wastes (bergamot, eucalypt, false pepper, laurel, pomegranate, and sage) were tested in *in vitro* against 10 soil-borne fungal pathogens at 2% (w/v). A resistance inducer product (EP5-product) was also used to improve plant resistance against the aggressiveness of fungal pathogens used. Based on the *in vitro* results, aliquots of bergamot, pomegranate, and EP5-product were applied to pots artificially inoculated with *Fusarium algeriensis*, *F. culmorum*, and *F. oxysporum* f.sp. *dianthi* and subsequently sown with wheat seeds to evaluate their *in vivo* inhibitory and phytotoxicity activities. Persistence was assessed by re-sowing the same soil after the first greenhouse trial. Phytosanitary status of the roots and the second leaf, expressed as phyto-toxicity, disease incidence, and plant growth features (seeds germination, plant height and dry-matter) were evaluated. Additionally, re-isolation of the inoculated fungal pathogens from the roots was determined. Bergamot, pomegranate and EP5-product showed the highest *in vitro* inhibitory activity. In the first greenhouse trial, EP5-product showed the greatest phyto-toxicity, though re-isolation frequency results indicated that it effectively protected the roots. In the second trial none of the PIPs showed phyto-toxic effects, but all significantly reduced MkI on roots and the second leaf. In this case, re-isolation frequency values were higher than in the first experiment, especially for bergamot. In conclusion, agricultural wastes as putative botanical fungicides, and the EP5-product are promising natural alternatives to chemical pesticides. The persistence of their efficacy, contrasted with the non-persistence of their phyto-toxicity, suggests that they are more suitable for soil biofumigation and less for foliar application.

1 | Introduction

It is known that global agricultural food demand is increasing by 35%–50% between 2012 and 2050, as the world population approaches 10 billion, placing increasing pressure on natural resources. Overall, reducing food loss and wastes could represent an essential strategy to lower production costs and improve the efficiency of the food system. To date the crop protection reflects the imperative to integrate natural and mineral substances to overcome the critical limitations of synthetic chemistry, in alignment with the European Green Deal (2030) objectives. The scientific pillars of this evolution include: (a) resistance management by natural substances operating through multi-site modes of action, significantly reducing selective pressure and the emergence of resistant pathogen strains typically associated with single-site synthetic molecules (García-Gutiérrez and Armenta-Reyes 2025); (b) agroecosystem resilience by the high selectivity and rapid biodegradability of

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these matrices preserving essential ecosystem services, protecting beneficial entomofauna (pollinators) and soil microbial biodiversity (Müller and Rossi 2024); and (c) toxicological safety by the absence of bioaccumulation and the minimization of chemical residues ensure high Food Safety standards while reducing chronic exposure risks for agricultural operators (European Food Safety Authority EFSA 2025). To adopt low-impact tools shifts plant protection from an exogenous control intervention to a systemic management of plant health, ensuring the economic and ecological sustainability of agricultural supply chains (FAO 2019; Smith and Zhao 2026). At the same time, consumers show growing interest in food produced through sustainable practices, including the reduction of chemical inputs to manage microbial diseases and preserve biodiversity. Indeed, agricultural crops are strongly affected by detrimental factors both abiotic and biotic origins, which reduce yields and limit global food availability (Oerke 2006). In general, pests, including fungi, bacteria, weeds, and insects, are strongly responsible of 20%–40% of global losses in agriculture (Savary et al. 2019). Indeed, fungal pathogens alone significantly reduce the yields of major crops by approximately 18% in wheat and 15% in rice, while combined losses from microbial pathogens, weeds, and animal pests can reach relevant values of 40% in crops such as maize and potato. The lack of adequate crop protection strategies, overall, may lead to 75% losses which could be reduced to about 40%, of which 21% reduction from bacterial and fungal pathogens, 8% from weeds, and 11% from animal pests (Oerke 2006). Pathogens and pests are major challenges for sustainable agriculture and environment protection, affecting food security and nutritional quality (Savary et al. 2017), although, chemical pesticides remain highly effective and widely accessible from farmers when rapid action is required to limit agricultural yield losses (Goulson 2014; Hayes et al. 2006; Mnif et al. 2011; Zheng et al. 2016). Conversely, many studies highlight that several chemicals are being progressively restricted or banned because of their environmental persistence (Alewu and Nosiri 2011; Suteu et al. 2020; Onwudiegwu et al. 2025), reduction of natural soil suppressiveness (Köninger et al. 2026; Sharma et al. 2023), residues in food products (Kumar et al. 2020), and toxicological effects for humans and livestock (Acosta-Lizárraga et al. 2026; Pathak et al. 2022). Their slow degradation and accumulation pose long-term risks to ecosystems and higher trophic levels (Zhou et al. 2025). Therefore, identifying sustainable alternatives to chemical pesticides, such as natural compounds and beneficial microorganisms, is an urgent priority for agricultural crops. Bio-pesticides derived from plants, microorganisms, insects, nematodes, or semiochemicals, represent environmentally sustainable and economically viable alternatives to synthetic pesticides (Tadesse Mawcha et al. 2025; Rahman et al. 2016). Recent studies discussed and confirmed the antifungal efficacy of several plant-derived products, including bergamot, pomegranate, sage, and other botanicals (Morea et al. 2025; Abo-Elyousr et al. 2025; Belgacem et al. 2021; Kulkarni et al. 2022; Romanazzi et al. 2016; Zaccardelli et al. 2020). Moreover, valorising agro-industrial by-products, such as residues from essential oil extraction or pomegranate peel from juice production, supports circular economy principles by reducing wastes and re-using them as natural sources (Morea et al. 2025; Prado et al. 2012). Wastes as agro-industrial by-products often contain un-known bioactive compounds with antifungal and antibacterial potentials (Tehranifar et al. 2011; Cebi and Erarslan 2023) that could reduce the yield losses with low management costs. As chemical pesticides have significant environmental and health impacts, in recent decades, the EU has progressively imposed stricter regulations on their use in agriculture. These regulations have driven research toward natural crop-protection alternatives, especially bio-products derived from agricultural wastes (AWs) and biocontrol agents. Indeed, many AWs derivatives from bergamot, pomegranate, and laurel, such as extracts, essential oils, and powders, have demonstrated strong potential against fungal pathogens tested both *in vitro* and *in vivo* (Cebi and Erarslan 2023; Laghezza Masci et al. 2024; Prado et al. 2012). This work focused on AWs used as putative inhibitory powders (PIPs) and included also the evaluation of resistance inducer product. The present study aims to evaluate plant-derived fungicidal products against soil-borne fungal pathogens affecting wheat, specifically assessing their effectiveness when used as minimal-process products (dry raw plant-derived products). In particular, this study aims to evaluate that minimally and eco-friendly processed plant products could be used in organic agriculture, without relying on organic-solvent extraction methods that are costly environment unfavourable. Therefore, the objectives are: (a) *in vitro* assessment of plant products derived from laurel, eucalypt, pomegranate, sage, bergamot, false pepper against ten soil-borne fungal pathogens, and a commercial resistance inducer for plants; (b) greenhouse evaluation of selected products as potential bio-fumigant; (c) and assessment of the residual antifungal effects of these products and their impacts on wheat agronomic traits.

2 | Materials and Method

2.1 | Preparation of Plants as Putative Inhibitory Products

Different plant parts presumed to possess potential antifungal activity (putative inhibiting products; PIPs) were collected from six botanical species: pomegranate (*Punica granatum* L.; peel) and sage (*Salvia officinalis* L.; leaves) collected from open field crops; eucalypt (*Eucalyptus camaldulensis* Dehnh.; leaves), laurel (*Laurus nobilis* L.; leaves) and false pepper (*Schinus molle* L.; leaves) collected from agricultural landscape in the Foggia province (Apulia region, Italy); bergamot (*Citrus bergamia* Risso & Poit.; peel) obtained as agro-industrial waste following essential-oil extraction from bergamot peel collected from agricultural landscape in Gioia Tauro (Calabria, Italy). After collection, the plant tissues were carefully washed, cut in small pieces and air-dried at room condition until approximately 50% weight loss. The semi-dehydrated plant tissues were put in stove at 30°C (FN 500 Sterilizer, Nuve, Ankara TR) until constant weight was reached. The plant PIPs were ground as finely as possible using an electric mill equipped with a fine-mesh screen (Geomil-50P) at 600 nm and stored in opaque plastic containers at darkens according to Morea et al. (2025). In addition, a commercial product (EP5-Protect), a resistance inducer for organic agriculture, was tested. Its formulation consists of minerals and silicates [citric acid (5%), iron (10%), zinc (6%), manganese sulphate (6.5% and bentonite (30%); Phenom Biotech srl, Milan Italy].

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2.2 | Fungal Soil-Borne Phytopathogens

To essay the ability of PIPs to inhibit fungal growth, ten soil-borne fungal pathogens of agronomic relevance were selected from the fungal collection of Plant Pathology and Diagnostic Laboratory of Department of Sciences Agriculture, Food, Natural Resources and Engineering (DAFNE) of University of Foggia, Italy: *Agroathelia rolfsii* (isolated from tomato; Apulia region), *Fusarium algeriensis* (isolated from wheat; Apulia region), *F. avenaceum* (isolated from tomato; Apulia region), *F. culmorum* (isolated from wheat; Apulia region), *F. oxysporum* (isolated from tomato; Apulia region), *F. oxysporum* f. sp. *dianthi* (*Fox* f. sp. *dianthi)* (isolated from wheat; Apulia region), *F. solani* (isolated from tomato; Apulia region), *Rhizoctonia solani* (isolated from grapevine; Apulia region), *Sclerotinia sclerotiorum* (isolated from broccoli; Apulia region), *Verticillium dahliae* (isolated from olive tree; Apulia region). In particularly, *A. rolfsii*, *S. sclerotiorum* and *V. dahliae* were previously essayed by Morea et al. (2025). Their taxonomic identification was carried out by morphological and molecular tools reported by Morea et al. (2025), using DNA extraction protocol described by Carlucci et al. (2013).

2.3 | Assessment of Putative Inhibiting Products (PIPs) Against Fungal Pathogens in In Vitro Conditions

Each plant powder and the EP5-product were incorporated into PDA medium (39 g/L; Sigma-Aldrich, Milan) at 2% (w/v). After adjusting the medium to pH 6.0, media were thermally treated at 115°C for 15 min in autoclave in order to avoid the reduction of the antimicrobial potential of PIPs. The inhibitory activity was performed on Petri dishes of 90 mm in diameter containing the PDA medium. Fungal plugs of 4.0 mm in diameter were excised from the margin of 14-day old colonies and placed at the center of each plate. Plates were incubated in darkness at 21 ± 3°C up to 21 days based on the mycelial growth of each fungal species in the control plates without PIPs. The experiment was replicated three times. The mycelial growth was detected every 48 h measuring perpendicular diameter axes. The inhibitory activity (IA; %) was calculated according to Carlucci et al. (2022) and Morea et al. (2025), as the percentage of mycelium growth inhibition compared to the control by the formula:

$$IA = \left(\frac{D_1 - D_2}{D_1}\right) \times 100$$

where D_1 was the measurement of the perpendicular diameter of the fungal colony on PDA control plates, and D_2 the measurement of the perpendicular diameter of the fungal colony on PDA plates enriched with PIPs powders.

The growth rates were also used to determinate the kinetics by a logistic equation modified by (Dantigny et al. 2011) as below reported:

$$D = \frac{D_{\max}}{1 + \exp[k(\tau - t)]}$$

Where D is the diameter of the colony over time; D_{max} is the maximum diameter of the fungal colony (55 mm); k is the rate of fungal growth (mm/day); τ is the time to attain a half of D_{max} (days) and t is the time (days). After this first step, a secondary modelling was performed as described by Bevilacqua et al. (2017). In this case τ was standardized as:

$$\Delta\tau = \tau_{PIP} - \tau_C$$

where τ_{PIP} and τ_C are the values from the media enriched with the PIPs and from the control plate, respectively.

2.4 | Essay of Bio-Fumigation Activity by PIPs in In Vivo Conditions

Based on the *in vitro* results obtained, bergamot, pomegranate and EP5-product were selected to test their effectiveness to remediate fungal infections within the soil. The putative bio-fumigation activity was set against *F. algeriensis*, *F. culmorum* and *F. oxysporum* f. sp. *dianthi* as fungal pathogens of wheat crop by artificial inoculation.

2.4.1 | *Artificial Fungal Inoculum Preparation*

The artificial inoculum of each fungal pathogen was prepared inoculating 100 g of wheat kernels (RGT voilur variety; Semetica, Bologna) preliminarily soaked in tap water in a 500 mL glass jar for 12 h. Then the water was removed and the kernels sterilized at 121°C for 20 min for three times. The sterilized kernels were inoculated with 5 mL of fresh fungal propagules suspension at concentration of $1{\cdot}10^5$ cfu/mL obtained by scraping with a scalpel the surface of a 14-days old fungal colony and collecting the propagules with a sterile solution (distilled H_2O; 0.5% Tween 20). All inoculated jars contained kernels inoculated by each fungal species were sealed with parafilm and incubated at 21°C ± 3°C in darkness and left to colonize for 21 days.

2.4.2 | *Setting up of Greenhouse Experiment*

For the greenhouse experiment, two layers of non-woven fabric were placed at the bottom of the 1.5 L pots to prevent the soil loss and covered with 5 cm of commercial Gaia soil (Agrochimica S.p.A., Bolzano). Twelve artificially inoculated kernels were placed on the moistened soil surface and covered with about 1 cm of soil. Then, the soil was watered to promote the growth of the fungal pathogen and covered with plastic film. After 72 h, aliquots of 10 g of powder from each PIP, supposed to have phyto-toxic effects for wheat seeds, were placed on the surface and covered with another 1 cm of the soil, and covered again with plastic film. Finally, after 72 h, 30 wheat seeds were sown at equal distance each other in the pot and covered with 1 cm of the soil. The experiment consisted of 16 different trials including the relative mocks, each with 16 replicates. Un-inoculated and un-treated pots were used as negative controls.

The germination rates of wheat seeds were recorded every 48 h until 240 h (10 days). Then, the phytosanitary status (consisting of leaf yellowing, as well as symptoms occurred on roots) was detected using an empiric patho-metric scale of seven classes to detected a putative phyto-toxicity damage, where 0= no symptoms observed; 1 = 1%–15%; 2 = 16%–35%; 3 = 36%–50%; 4 = 51%–70%; 5 = 71%–85%; 6 = 86%–100%. The phyto-toxicity

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damages occurred on the roots were determined as phyto-toxicity index (PTxI) according to the following formula:

$$PTxI\ (\%) = \frac{\sum(N_n\ x\ C_n)}{N_{tot}\ x\ C_{\max}}\ x \quad 100$$

where N_n is the number of cases observed for each class, C_n is the reference class, N_{tot} is the total number of cases observed, and C_{max} is the highest class of the scale.

Other features, such as second leaf status and plant length (including roots) of survived young wheat seedlings were monitored once a week. After 45 days, the wheat plants were gently removed from the pot, and the roots were carefully washed and subjected to empiric patho-metric scale as described above in order to assess putative phyto-toxicity damages. Then, the wheat roots were subjected to mycological analyses to ascertain the presence of the inoculated fungi as re-isolation frequency (R-IF; %). At the end of plant cycle, dry-matter (%) was also determined according to the following formula:

$$Dry\ matter = \frac{Dry\ sample\ weight \quad (g)}{Wet\ sample\ weight \quad (g)}\ x\ 100$$

2.5 | Assessment of Persistency of PIP Activity as Putative Bio-Fungicide Against Fungal Pathogens in In Vivo Conditions

Based on the results obtained from the bio-fumigation experiment, the next experiment was performed to evaluate the persistency of PIPs into the soil as putative bio-fungicidal products against the artificially inoculated fungal pathogens. For this purpose, the same pots, containing the same soil from the first experiment, were used to sow 30 wheat seeds of the same variety above-mentioned. The methodologies to detect germination rates, general phytosanitary status, plant vigour and laboratory analyses were the same performed during the first experiment. Therefore, the experiment consisted of 16 different trials including the relative mocks, each with eight replicates. Un-inoculated and un-treated pots were used as negative controls. The data regarding the wheat seed germination and the phytosanitary status of wheat plants were collected each 2 days, while the height of the plants was measured once a week. After 45 days, the plants were subjected to laboratory analyses regarding the symptoms occurred on roots, stem and leaves, and plant growth indices. The phytosanitary status of wheat roots was detected using the same empiric patho-metric scale of seven classes to detected putative phyto-toxicity damage above-mentioned. The disease damage occurred on roots, expressed as disease incidence by McKinney index (MkI; %), was determined according to the following formula:

$$MkI\ (\%) = \frac{\sum(N_n\ x\ C_n)}{N_{tot}\ x\ C_{\max}}\ x \quad 100$$

where N_n is the number of cases observed for each class, C_n is the reference class, N_{tot} is the total number of cases observed, and C_{max} is the highest class of the scale.

2.6 | Re-Isolation of Artificially Inoculated Fungal Pathogens

Portions of plant root were used to perform fungal isolation detecting the presence of the inoculated fungal pathogens. Roots of each plant were gently washed with distilled water to remove the soil and cut into pieces of almost 1.0 cm. The surface of the tissue pieces was disinfected using subsequent solutions of 70% ethanol, 20% sodium hypochlorite and 70% ethanol again for 30 s each. Plant tissue pieces were additionally washed with sterilized distilled water for 30 s to remove any trace of sodium hypochlorite and ethanol according to Fisher et al. (1992). Sterilized pieces were dried in a sterile filter paper disc under laminar flow cabinet util they were completely dried. For each trial, six pieces were placed on the surface of PDA with 500 ppm of streptomycin (Sigma-Aldrich, Milan). Two plates were prepared for each trial.

2.7 | Statistical Analyses

The statistical analyses were performed by JMP software package, version 16.2 (SAS Institute Inc., Cary, NC, USA) on the datasets collecting during all experiments (inhibition, bio-fumigation and PIPs persistence activities). The percentage data of both PIPs activities (IA; PTxI; MkI) were arcsine root-square transformed in Excel 2007 by the formula DEGREES(ASIN (SQRT(X))), where X is the percentage value.

To test if the in vitro and in vivo datasets followed normal distributions, the Shapiro-Wilk test (W test) was used. Homogeneity of variance of datasets on the base of the fungal species (ten in in vitro experiment; three in in vivo experiment) and the PIPs (seven in in vitro experiment; three in in vivo experiment) were assessed using the Levene test. One-way and Factorial ANOVA analyses were performed to determine the significance of difference on IA, PTxI and MkI by PIPs against fungal growth in in vitro and in vivo conditions, the differences due to sensitivity of each fungus essayed, and to detect any interaction between these factors (Fungus × PIP). One-way ANOVA was performed to determine the significant differences in: inhibitory activity (IA) caused by each fungal species tested, and any difference due to the seven PIPs used in in vitro conditions; phyto-toxicity activity (PTxI), and disease incidence (MkI) by PIPs and fungi in in vivo conditions, respectively. Tukey's tests were used for the comparison of the treatment means, at $p < 0.01$.

To assess the significant differences regarding the *in vivo* datasets of plant length and dry-matter were also statistically analysed performing One-way and Factorial ANOVA analyses taking into account the fungal pathogen and the PIP using JMP, version 16.2 (SAS Institute Inc., Cary, NC, USA).

3 | Results

3.1 | Assessment of Inhibitory Activity by Putative Inhibiting Products Against Fungal Pathogens in In Vitro

According to Shapiro–Wilk test, the inhibitory activity (IA) data followed normal distributions for all fungal pathogens and PIP, with W value of 0.89 ($p < 0.0001$). Levene test showed that the homogeneity of variance was also significant for the fungal

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pathogens and PIPs (F = 21.91/10.59; $p < 0.0001$). One-way and Factorial ANOVA analyses demonstrated that significant differences in inhibitory activity (IA) played by the PIPs against fungal pathogens were observed.

Figure 1, grouping four panels (a-e), presents the results of the *in vitro* assay evaluating the growth inhibition of fungal pathogens when exposed to various putative inhibiting products (PIPs), as well as PIPs efficacy. Panel (a) (Table S1) reports data regarding the sensitivity of fungi *versus* PIPs activity, where it is possible to observe that, in general *A. rolfsii* was the least inhibited by all PIPs treatments, while *R. solani* was the most sensitive, with IA mean values of 4.30% and 68.05%, respectively. The IA values, expressed as least squares means, show that the PIPs were moderately significant in inhibiting the growth of *F. algeriensis, F. culmorum, F. oxysporum* f. sp. *dianthi* and *F. solani* (IAs of 64.50%, 50.65%, 37.81% and 36.38%, respectively). No significant IA value was recorded regarding *S. sclerotiorum* (27.92%) and *F. avenaceum* (15.85%). Regarding the effectiveness of PIPs to inhibit all tested fungi, panel (b) (Table S2) compares PIPs IA values as means of minimal squares showing that EP5-product (IA = 73.53%) was by far the most effective to inhibit fungal growth, followed by pomegranate (IA = 58.14%), eucalypt (IA = 52.45%) and bergamot (IA = 44.41%). While sage, laurel and false pepper showed the lowest IA mean values ranging from 21.00% to 15.27% (Figure 2). In particular, panel (c) (Table S3) reports all interactions among PIPs and fungal pathogens with specific IA values obtained (ranging from 93.75% to 100.00%) highlighting that *F. algeriensis*, *F. culmorum, F. oxysporum, S. sclerotiorum* and *V. dahliae* were strongly inhibited by EP5-product; *R. solani* and *V. dahliae* by pomegranate; and *R. solani* by both bergamot and eucalypt. Among the fungal pathogens, *F. avenaceum* was least sensitive to IA by PIPs, as it was significantly inhibited only by EP5-product with IA value of 80.07%, not significantly inhibited by eucalypt (9.09%) and pomegranate (21.82%), and not inhibited at all by bergamot, false pepper, laurel and sage (0.00%). Moreover, *A. rolfsii* was clearly the fungal pathogen less sensitive to IA by PIPs, because EP5-product, eucalypt, sage, laurel and false pepper produced no inhibition, and not significant IA values were obtained by bergamot (12.73%) and pomegranate (15.45%). Panel (d) (Table S4) reports the growth kinetics influenced by the cumulate effect of PIPs: EP5-product ($\Delta\tau = 204.27$ h), eucalypt ($\Delta\tau = 86.42$ h), pomegranate ($\Delta\tau = 75.02$ h) and bergamot ($\Delta\tau = 45.12$ h) showed a significant positive $\Delta\tau$, suggesting that they could reduce the speed of fungal growth, confirming their high antifungal capacity. Also in this analysis EP5-product has been the most effective among the other PIPs essayed, as it reduced the growth speed with a $\Delta\tau$ greater than 200 h.

3.2 | Essay of Bio-Fumigation Activity by PIPs in Greenhouse Condition

Figure 3, grouping seven panels (a-g), illustrates the effects of various treatments on wheat germination and root health under fungal pathogen stress and PIP application in the greenhouse experiment. All panels of Figure 3 analyse different aspects of treatment impact, such as germination rates, PIP phyto-toxicity as rot and browning root, and reduced plant growth. Panel (a) (Table S5) reports that no significant effect on germination rates of wheat seeds from inoculated fungi was detected because they were similar across the trials. Panel (b) (Table S6), regarding the effect of PIPs on germination rates on wheat seeds, highlights that bergamot and pomegranate did not reduce the rates of wheat germination compared to the trial un-treated with PIPs (90.94%). In contrast, EP5-product reduced significantly the wheat germination rates (32.50%) compared to the un-treated trial. Panel (c) (Table S7), illustrating the interaction effects of fungal pathogens and PIPs on germination rates of wheat seeds, confirms that plant PIPs did not exert any inhibiting effect on wheat germination, but a significant putative phyto-toxic effect was caused by EP5-product treatment in all trials, reducing the germination rates of wheat seeds to values ranging from 29.17% to 35.21%. Moreover, no cumulative effects have been observed by both fungal inoculations and PIP treatments. Panels (d-f) of Figure 3 report the results from One-way and Factorial ANOVA analyses on data collected from symptoms observed on roots of wheat plants at the end of the experiment in greenhouse conditions, as mean of minimal squares, where the wheat seeds were treated with both fungi and PIPs (Figure 2i–k). Panel (d) (Table S8) highlights only the effect of fungal presence on wheat plants treated and un-treated with PIPs, expressed as putative phyto-toxicity incidence (PTxI; %). PTxI was higher when the wheat plants were artificially inoculated with *F. algeriensis* (PTxI = 60.16%), *F. culmorum* (PTxI = 55.64%) and *F. oxysporum* f. sp. *dianthi* (PTxI = 54.47%) compared to trial that was un-treated with fungi but treated only with PIPs, although phyto-toxicity symptoms were still observed (PTxI = 41.92%), likely due to native fungi associated with wheat seeds used in the *in vivo* experiment. Panel (e) (Table S9) reports the putative phyto-toxic effects of PIPs on wheat roots or their antifungal activity against artificially inoculated fungal pathogens. Based on the results presented in panel (e), the most effective PIPs were bergamot and pomegranate with lower putative phyto-toxicity values of 39.58% and 42.58%, respectively. Instead, EP5-product significantly affected the wheat roots, with putative phyto-toxicity value of 61.23%. In contrast, the roots of un-treated wheat plants with any PIP, but treated and un-treated with fungal pathogens, were more affected, with a disease incidence value of 68.80%. Moreover, panel (f) (Table S10) reports the combination of fungal pathogen and PIP effects on wheat roots, showing that bergamot and pomegranate were slightly more effective in controlling fungal species, with PTxI values ranging from 31.79% (bergamot *vs F. oxysporum* f.sp. *dianthi*) to 49.41% (pomegranate *vs F. algeriensis*) compared to un-treated trials by any fungus (47.79%). Also in this case, it seems that the treatment with EP5-product was not effective because PTxI values were higher (69.03%, in presence of *F. oxysporum* f.sp. *dianthi*; 66.80%, in presence of *F. algeriensis*; and 67.49% in presence of *F. culmorum*) than trial un-inoculated with fungi (PTxI = 47.79%), although lower than trials un-treated with any PIP. Panel (g) (Table S11), reporting the re-isolation frequency percentage (R-IF; %) of artificially inoculated fungi, highlights that EP5-product allowed to record the lowest R-IF, ranging from 0.00% to 5.30%, respect to other PIPs. The same panel highlights that between the other PIPs, bergamot was slightly more effective to inhibit the fungal growth (from 8.30% to 18.7%) than pomegranate (from 14.60% to 19.7%). In contrast, R-IF values of artificially

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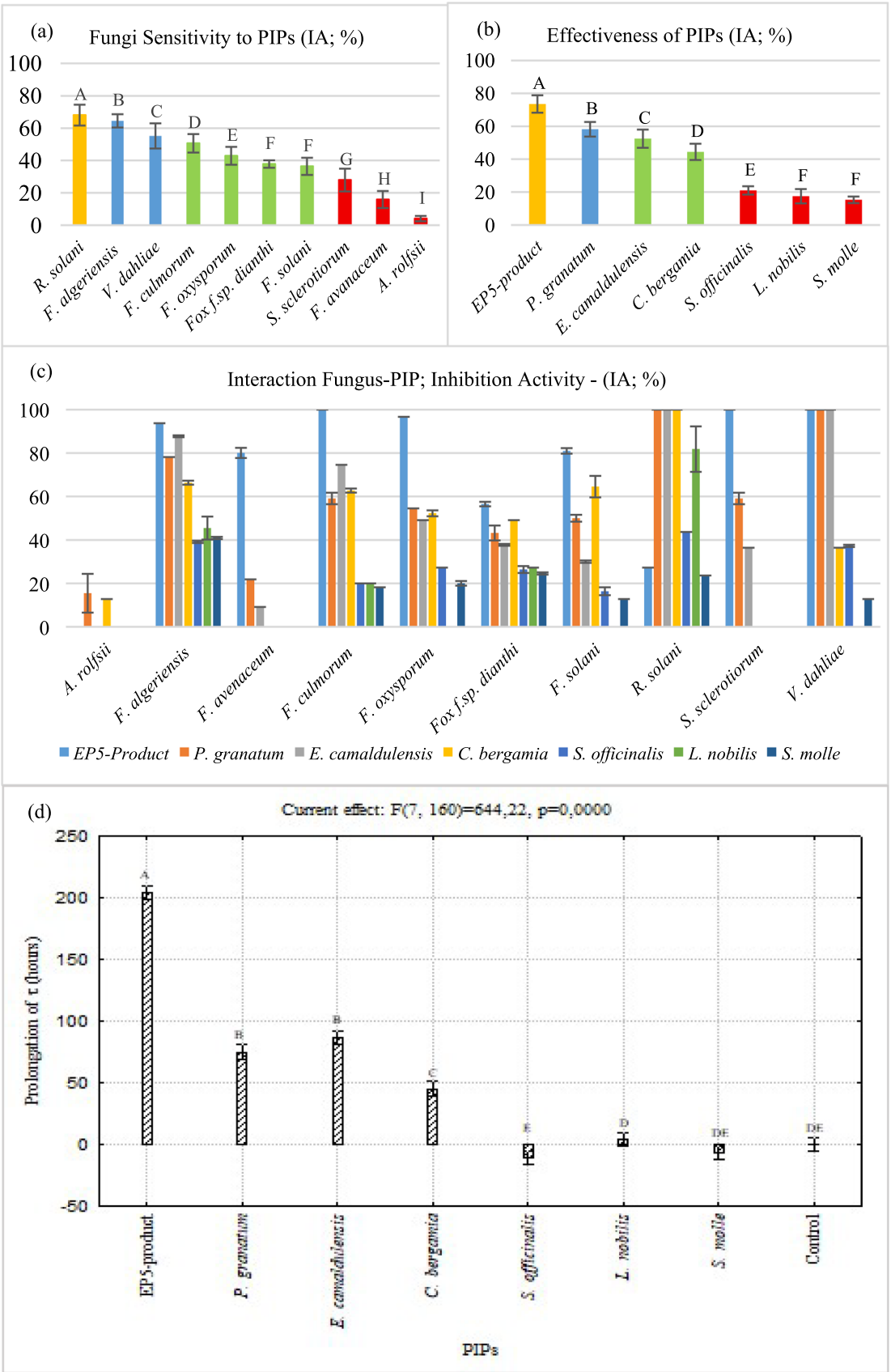


FIGURE 1 | Legend on next page.

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inoculated fungi related to the control trial were higher than trials treated by PIPs, ranging from 22.80% (in presence of *F. oxysporum* f.sp. *dianthi*) to 27.10% (in presence of *F. algeriensis*).

Figure 4 illustrates the effects of fungal pathogens, PIPs, and their combined treatments on the phytosanitary status of the second leaf, as well as on the growth parameters (plant length and dry-matter) of wheat plants under greenhouse conditions by nine panels (a-i). Panel (a) (Table S12) highlights that the presence of fungal pathogens negatively affected the health of the second leaf, which likely showed symptoms such as chlorosis, necrosis, or reduced leaf area. This reflects the direct pathogenic impact on leaf tissues, leading to impaired photosynthetic capacity and overall plant vigour. Indeed, the putative phyto-toxicity incidence (PTxI; %), expressed as mean of minimal squares, occurred on second leaf of wheat plants, was statistically significant (ranging from 62.92% to 80.73%) when the fungal pathogens were artificially inoculated compared to the un-treated trial (PTxI = 35.56%). In contrast, panel (b) (Table S13) reports that the application of EP5-product produced a significant phyto-toxic effect on leaf (PTxI = 71.19%), while bergamot and pomegranate slightly improved leaf health (PTxI ranging from 58.21% to 60.19%), indicating a lack of phyto-toxic effect by themselves, and a possible bio-stimulant or protective action respect to un-treated wheat plants (PTxI = 70.00%). Panel (c) (Table S14) illustrates the interaction of treatments (fungus × PIP), where the degree of leaf damage was reduced from PIP treatments compared to fungal pathogen-only treatments, although a slightly minor protective effect was observed in trials treated with EP5-product (PTxI = 49.45%–87.92%).

Similar results were recorded from wheat plant length (cm), where the presence of artificially inoculated fungi did not influence it (panel d) (Table S15), while all PIP treatments reduced it, although EP5-product was the most phyto-toxic (26.3 cm) respect to un-treated trial (31.57 cm) (panel e) (Table S16). The interaction fungus × PIP confirmed that inoculated fungus did not impact directly the length of wheat plants, while pomegranate (from 28.59 to 29.99 cm) and EP5-product (from 25.94 to 26.67 cm) showed potential phyto-toxic effects respect to trial treated with bergamot (from 30.27 to 33.75 cm) and un-treated (from 30.38 to 32.85 cm) (panel f) (Table S17). Regarding the effect of inoculated fungi (panel g) (Table S18) and PIPs (panel h) (Table S19), and their interaction (panel i) (Table S20) on dry-matter (%) obtained from entire wheat plants at the end of the greenhouse experiment, the trends were similar to those described for the plant lengths, showing very low significant results. Indeed, it seems that neither substantial decrements by inoculated fungi nor benefits from use of PIPs were observed.

3.3 | Assessment of Persistency of PIPs as Residual Activity Against Fungal Pathogens Through the Second Greenhouse Experiment

Figure 5 summarizes how different PIP treatments and their residual effects influenced wheat seeds germination, roots symptoms, and fungal pathogens persistence. In particular, it illustrates the effect of residual activity by PIPs on both wheat seeds germination rates and roots phytosanitary status of wheat plants through the results presented by the seven panels (a-g) regarding specific aspects of the study occurred at the end of the second greenhouse experiment, as means of minimal squares by One-way and Factorial ANOVA analyses. Panels (a) (Table S21) and (b) (Table S22) highlight the surviving of artificially inoculated fungi and the persistence of efficacy of PIPs applied during the first greenhouse experiment, and their influence on the germination rates of wheat seeds. In particular, no significant effect was observed due to fungal presence (panel a), while the PIPs used played a significant residual activity, slightly increasing the germination rates compared to the un-treated trial (panel b). The same trend is visible in panel (c) (Table S23), where the germination rates were significantly affected only from persistent fungal pathogens inoculated in trials un-treated with PIPs. Regarding the phytosanitary status of wheat roots, panels (d-f) illustrate that the root exhibited significant symptom reduction with MkI values ranging from 19.98% (inoculated with *F. culmorum*) to 8.57% (un-treated trial) (panel d) (Table S24) respect to the results described in Figure 3 panel (d) where MkI values ranged from 60.16% (in presence of *F. algeriensis*) to 41.92% (un-treated trial). Likewise, panel (e) of Figure 5 (Table S25) shows that the putative persistence of antifungal activity by PIPs significantly reduced the MkI values from 28.03% (un-treated trial) to 4.73% (trial treated with EP5-product) respect to those described in panel (e) of Figure 4, which ranged from 68.80% (un-treated trial) to 39.58% (treated trial with bergamot). The effect of interaction between PIP treatments and fungal pathogens reported in panel (f) (Table S26), regarding the phytosanitary status on wheat roots, reports that EP5-product was the most persistent and effective in reducing the damages caused by inoculated fungi, with MkI values ranging from 5.95% (*F. culmorum*) to 2.42% (*F. oxysporum* f. sp. *dianthi*) respect to trial inoculated only with fungi and un-treated with any PIP, where MkI values ranged from 40.48% (trial inoculated with *F. culmorum*) to 8.79% (un-inoculated and un-treated trial) (Figure 2–n). These results demonstrated that fungal pathogens inoculated during the first greenhouse experiment could survive in soil until the end of the second experiment. Similar trend was observed in remaining trials, since persistency effect of PIPs continued to significantly inhibit the surviving fungi, for both bergamot, with MkI values ranging from 7.59% (un-treated trial) to 13.80% (in presence of *F. algeriensis*), and pomegranate, with MkI values ranging from 4.48% (in presence of *F. oxysporum* f.sp. *dianthi*)

FIGURE 1 | *In vitro* evaluation of fungal pathogen sensitivity and inhibition by putative inhibiting products (PIPs): (a) sensitivity of different fungal pathogens *versus* PIPs expressed as inhibitory activity (IA; %); (b) comparative efficacy of the PIPs against the fungal pathogens showing their relative antifungal potential (One-way ANOVA, Tukey Test, $p < 0.01$); (c) interaction effects between fungal pathogens and PIPs (EP5-product, *Punica granatum*, *Eucalyptus camaldulensis*, *Citrus bergamia*, *Salvia officinalis*, *Laurus nobilis* and *Schinus molle*) expressed as IA percentages (Factorial ANOVA, Tukey Test, $p < 0.01$); (d) hypothesis decomposition of the multi-factorial ANOVA highlighting the significant effect of PIP type on inhibitory activity. The means with the same capital letters indicate not significant differences.

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FIGURE 2 | Inhibitory activity (IA) played by the putative inhibiting products (PIPs) in *in vitro*: (a) growth of *Verticillium dahliae* in the control plate; (b) IA played by *Punica granatum* against *V. dahliae*; (c) growth of *Fusarium algeriensis* in the control plate; (d) IA played by EP5-product against *F. algeriensis*; (e) growth of *F. oxysporum* f. sp. *dianthi* in the control plate; (f) IA played by *Citrus bergamia* against *F. oxysporum* f.sp. *dianthi*; (g) growth of *F. avenaceum* in the control plate; (h) IA played by *Schinnus molle* against *F. avenaceum*. Phyto-toxicity (PTxI) played by the PIPs *in vivo:* (i) un-treated roots after the first essay; (j) roots inoculated with *F. algeriensis* after the first essay; (k) roots treated with EP5-product after the first essay; (l) un-treated roots after the second essay; (m) roots inoculated with *F. culmorum* after the second essay; (n) roots treated with EP5-product after the second essay.

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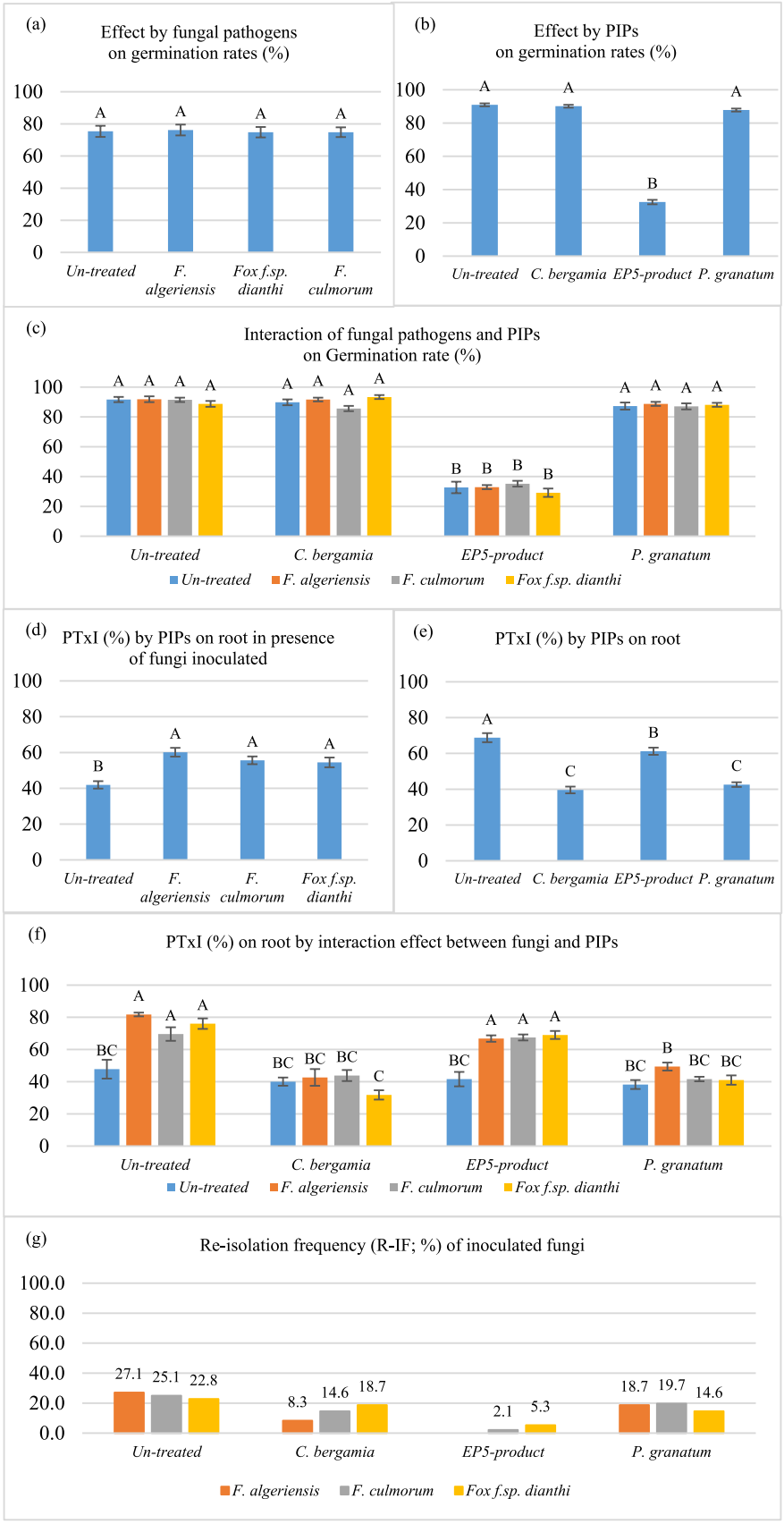


FIGURE 3 | Effects on wheat seeds germination rates and phyto-toxicity (PTxI; %) by the putative inhibiting products (PIPs) in the greenhouse experiment: (a) effect of fungal pathogens; (b) effect of PIPs; (c) effect of interaction between fungi and PIPs; (d) putative phyto-toxicity by PIPs on wheat roots in presence of inoculated fungal pathogens; (e) phyto-toxic activities by PIPs on wheat roots; (f) root phyto-toxicity induced by interaction of treatments (fungi *vs* PIPs); (g) percentage of inoculated fungal pathogens re-isolated from wheat roots. One-way and Factorial ANOVA (interaction fungi *vs* PIPs), Tukey Test, $p < 0.01$ were performed; the means with the same capital letters indicate not significant differences.

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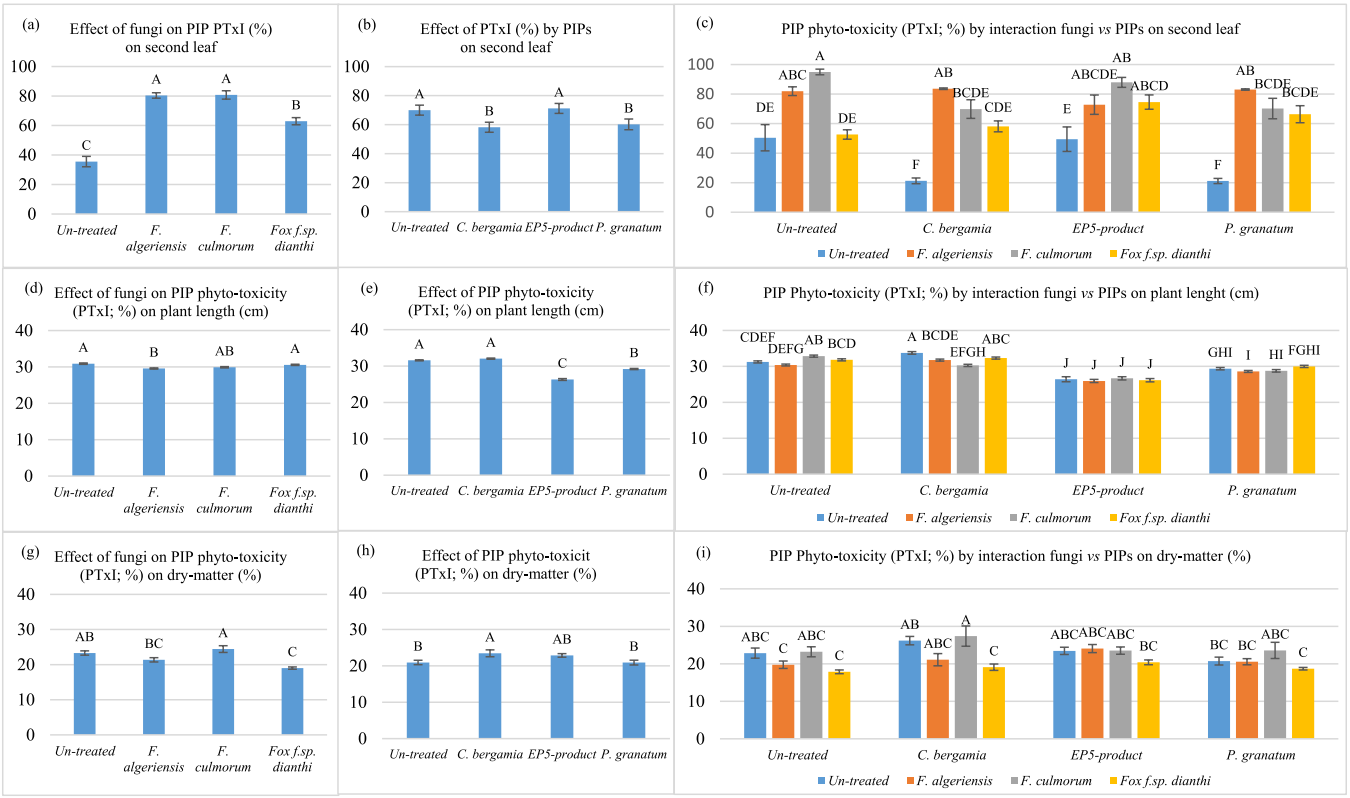


FIGURE 4 | Effect of putative phyto-toxicity (PTxI) from inhibiting products (PIPs) treatments on the phytosanitary status of the second leaf and on the growth indices of wheat plants: (a) effect of fungal pathogens on PIP phyto-toxicity occurred on the second leaf; (b) putative phyto-toxic effect of PIPs on the second leaf; (c) putative phyto-toxic effect of the treatments by interaction between fungus *vs* PIP on the second leaf; (d) effect of fungal pathogens on PIP phyto-toxicity occurred on plant length; (e) putative phyto-toxic effect of PIPs on plant length; (f) putative phyto-toxic effect of PIPs by interaction between fungus *vs* PIP on plant length; (g) effect of fungal pathogens on dry-matter; (h) effect of PIPs on dry-matter; (i) effect of the interaction of both treatments (fungus *vs* PIP) on dry-matter. One-way and Factorial ANOVA (interaction fungus *vs* PIP), Tukey Test, $p < 0.01$ were performed; the means with the same capital letters indicate not significant differences.

to 21.35% (in presence of *F. culmorum*). These results are confirmed by re-isolation analyses (panel g) (Table S27) because the re-isolation frequencies of surviving fungi were very low in the trial treated with EP5-prdoduct (7.31%–12.58%). Respect to EP5-product, the other PIPs showed to be still able to inhibit the fungal pathogens, since they reduced the R-IF of inoculated fungi with values ranging from 41.67% to 58.33% by bergamot, and from 22.92% to 52.08% by pomegranate, highlighting a significant persistency into soil until the end of the second greenhouse experiment. Indeed, the R-IF values of inoculated fungi were significantly higher in the trial un-treated with any PIP (41.67%–87.50%).

Figure 6 (panels a-i) presents the residual effect of PIPs treatments and effect of surviving inoculated fungi on the phytosanitary status of second leaf and on the plant's growth index. The residual antifungal activity by bergamot, pomegranate and EP5-product reduced disease incidence (MkI) values of 70.86, 68.97, and 42.80% (panel b) (Table S29), respectively. As for the other indexes, EP5-product gave the best results, with the smallest yellowing leaf surface among the treatments. Although bergamot and pomegranate gave similar results, in this experiment, the un-inoculated trial treated with bergamot produced the highest MkI value (82.6%) respect to the trials inoculated with the fungi (panel c) (Table S30), suggesting that maybe in this case a residual phyto-toxic effect on the second leaf may be occurred. In the second greenhouse experiment, the effect of the surviving fungal pathogens and the persistent effect of PIPs on the plant length were more relevant. *Fusarium algeriensis*, *F. culmorum*, and *F. oxysporum* f.sp. *dianthi* slightly limited the wheat plant length, with values of 28.61, 28.42, and 27.71 cm, respectively (panel d) (Table S31). Among the PIPs, pomegranate and EP5-product promoted significantly the plant length with values of 29.06 and 28.88 cm, while bergamot limited it, although not significantly, with a value of 27.87 cm (panel e) (Table S32). The length of wheat plants treated with EP5-product (range 27.40–30.11 cm) and pomegranate (range 28.35–30.38 cm) was slightly favoured respect to the plants treated with bergamot (range 26.61-29.60 cm) and un-treated (range 25.50-29.46). In contrast, the dry-matter determined through all trials was not affected by the presence of the surviving fungal pathogens, but it was by the effect of residual persistence of PIPs. While no significant difference was observed among the inoculated fungal pathogens (panel g) (Table S34), the trials treated with pomegranate showed higher dry-matter value (30.53%) than the ones treated with EP5-products, bergamot, and the un-treated control, with values of 25.10%, 26.73%, and 26.10%, respectively (panel h) (Table S35). Overall, the un-inoculated wheat plants treated with pomegranate were the ones with the highest value. In the remaining trials, the un-treated wheat plants and the ones inoculated with the same fungal pathogen showed similar dry-matter values, except for the ones treated with bergamot and inoculated with

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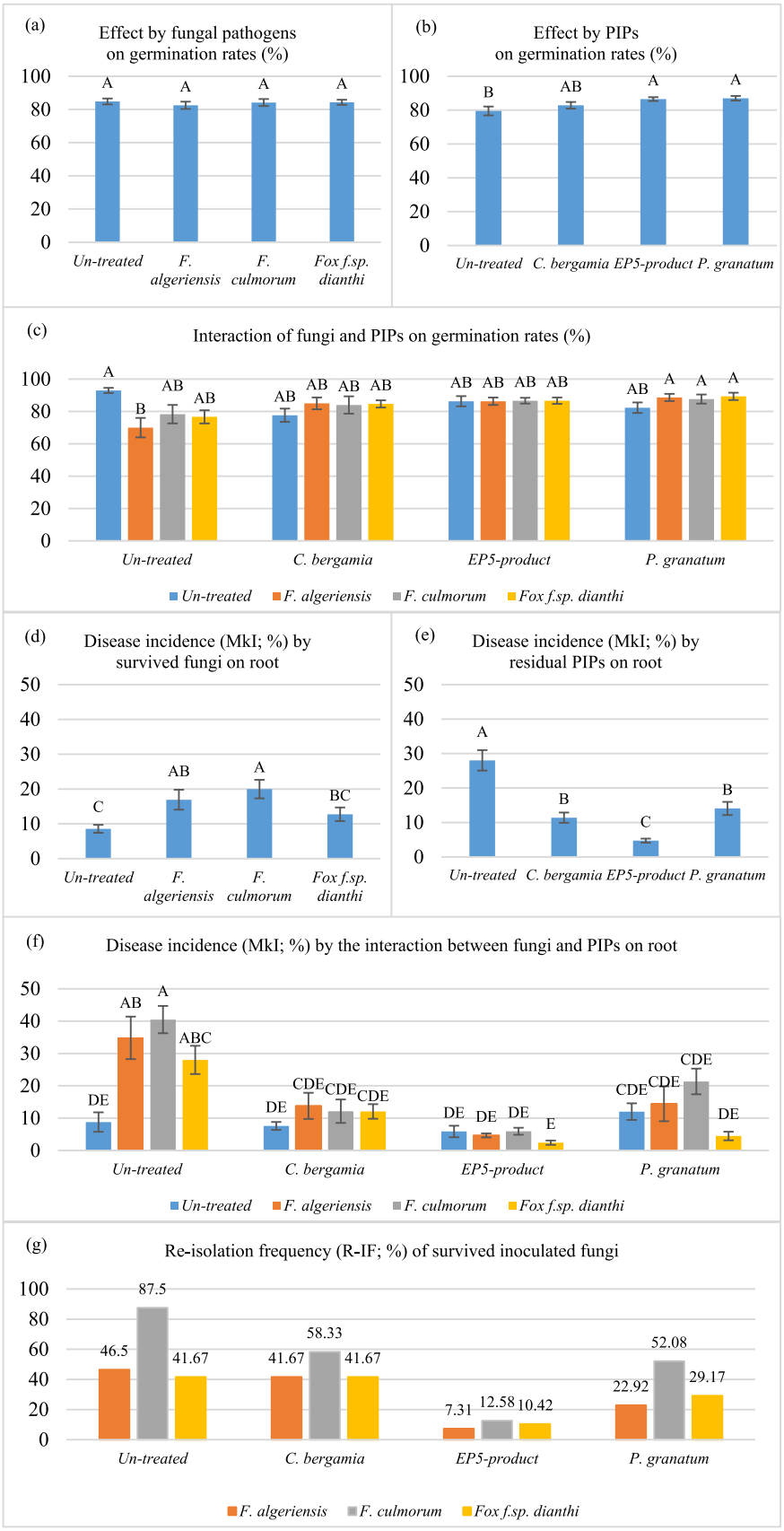


FIGURE 5 | Legend on next page.

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F. algeriensis, where the dry-matter value was slightly higher (panel i) (Table S36).

4 | Discussion and Conclusion

The selected PIPs and the commercial product were tested against ten soil-borne fungal pathogens *in vitro* to select the best candidates for greenhouse experiments. The results obtained in *in vitro* conditions collectively demonstrate that the inhibitory effects from PIPs used in this study are both fungus-dependent and PIP-specific dependent. EP5-product consistently exhibited the highest antifungal performance across nearly all fungal pathogens, followed by pomegranate, eucalypt and bergamot, although with varying selective efficacy. The kinetic data reinforce the static inhibition results, confirming that EP5-product not only inhibits fungal growth but also slows fungal development over time. These findings suggest that EP5-product is a promising candidate for biocontrol applications against a broad range of phytopathogenic fungi, whereas other PIPs may be useful in targeted management strategies depending on the pathogen involved, because their antifungal activity observed in *in vitro* essays was variable. Carlucci et al. (2024) and Morea et al. (2025) also reported high efficacy of EP5-product against several fungal pathogens, such as *Agroathelia rolfsii*, *Botrytis cinerea*, *Fusarium avenaceum*, *F. oxysporum*, *Monilia laxa* and *Verticillium dahliae*. The putative efficacy of this PIP is mainly due to the presence of mineral and silicates that create unfavourable conditions for fungal growth (Morea et al. 2025) reinforcing the consideration that natural compounds such as zeolite (similar to EP5-protect) and other similar materials might be largely used in agriculture in according to Ahumada-Rudolph et al. (2025). Our results regarding the silicate-rich EP5-product are supported by recent studies showing that zeolite and bentonite mitigate soilborne root diseases in horticulture plants. These minerals act as slow-release reservoirs for water and silicon; the latter accumulates in root cell walls to provide a physical barrier against fungal infections (Cataldo et al. 2021). This study also observed variability in fungal species, highlighting differential sensitivity to the antifungal activity of PIPs used, in according to several studies regarding the same topics. Many studies have demonstrated the ability of pomegranate, eucalypt and bergamot to reduce linear growth of several pathogens using their organic-solvent derivates. Mohamad and Khalil (2015) evaluated the antifungal activities of the methanolic extract of pomegranate peel against different phytopathogenic fungi. Maj et al. (2024) discussed interesting results obtained during their investigation of natural dry plants (including *Eucalyptus* sp.) and derivates (such as oils) against twenty different isolates of *Neosartorya* spp. concluding that they were able to inhibit and alter the fungal growth and metabolism. These results align with other investigations demonstrating that pomegranate methanolic extract almost completely inhibits the linear growth of *R. solani* and moderately *Fusarium* spp. In addition, Antón-Domínguez et al. (2023) tested extracts obtained using ethanol, methanol, or ethyl acetate as solvents at various doses against *V. dahliae*. Extracts obtained with ethanol and methanol significantly reduced the viability of *V. dahliae* structures when applied at the highest dose, whereas ethyl acetate was not effective at any dose. Pomegranate peel extracts were also effective in controlling the fungal pathogens in *in vivo* conditions when applied by irrigation. Al-Askar (2012) investigated the antifungal activities of the ethanolic extract of the pomegranate peel against five fungal pathogens at different concentrations, showing slight efficacy against *A. rolfsii*. In a study regarding the application *in vitro*, Katooli et al. (2011) evaluated the antifungal activity of eucalypt essential oil against several fungal pathogens, with *R. solani* exhibiting complete inhibition of mycelium growth. Tanovic et al. (2007) tested 17 commercial essential oils against soil-borne fungal pathogens. Among them, bergamot essential oil limited significantly the growth of *R. solani*, and moderately the growth of the *F. oxysporum*.

Based on the *in vitro* results, bergamot, EP5-product, and pomegranate dry powders were tested to confirm their efficacy against *Fusarium algeriensis*, *F. culmorum* and *F. oxysporum* f.sp. *dianthi* in *in vivo* conditions by performing two greenhouse essays. In the first one, high amounts of each PIP (ten grams per 1 L of total volume) were applied to assess their putative ability to inhibit artificially inoculated fungi, simulating a bio-fumigation practice. The results demonstrated that bergamot and pomegranate PIPs exhibited mild antifungal effects and no phyto-toxicity, suggesting suitability as biocontrol or protective agents, but not as bio-fumigants. In contrast, EP5-product showed strong phyto-toxic effects, including reduced wheat seed germination, presence of phyto-toxic damages on root, and high fungal inhibition, as evidenced by nearly zero fungal re-isolation frequencies. Phyto-toxicity from EP5-product was also observed on second leaf damage, reduced wheat plants length, and decreased dry-matter of wheat plants. These results indicate that high quantities of EP5-product could be used as a bio-fumigant before sowing and/or seedling transplanting, when used at high dosage levels, for fungal pathogen suppression and weed control. EP5-product phyto-toxicity appears to be related to its composition, and in particular at its citric acid content. Indeed, Agnello et al. (2015) tested citric acid effect on the germination of alfalfa (*Medicago sativa* L.) seeds, resulting able to reduce their germination. Anyway, since no studies on bio-fumigation by EP5-product or by similar minerals and silicates are available, further investigations are ongoing (data not available). The evaluation of PIP persistence after 45 days set up on second greenhouse experiment demonstrated a lasting suppressive effect of PIPs on inoculated fungal pathogens and root disease development in wheat plants. In particular, fungal

FIGURE 5 | Effect of the residual activity from putative inhibiting products (PIPs) treatments on germination and root phytosanitary status of the wheat plants: (a) effect of the probable presence of survived fungal pathogens on germination rates; (b) effect of the residual activity by PIPs on germination rates; (c) effect from interaction fungi × PIP treatments on germination rates; (d) root symptoms caused by surviving fungal pathogens; (e) root symptoms due to the residual PIP activities; (f) effect from interaction fungi × PIP treatments in affecting root health; (g) percentage of inoculated fungal pathogens re-isolated from wheat roots indicating fungal infection persisted or was suppressed. One-way and Factorial ANOVA (interaction fungi × PIPs), Tukey Test, $p < 0.01$ were performed; the means with the same capital letters indicate not significant differences.

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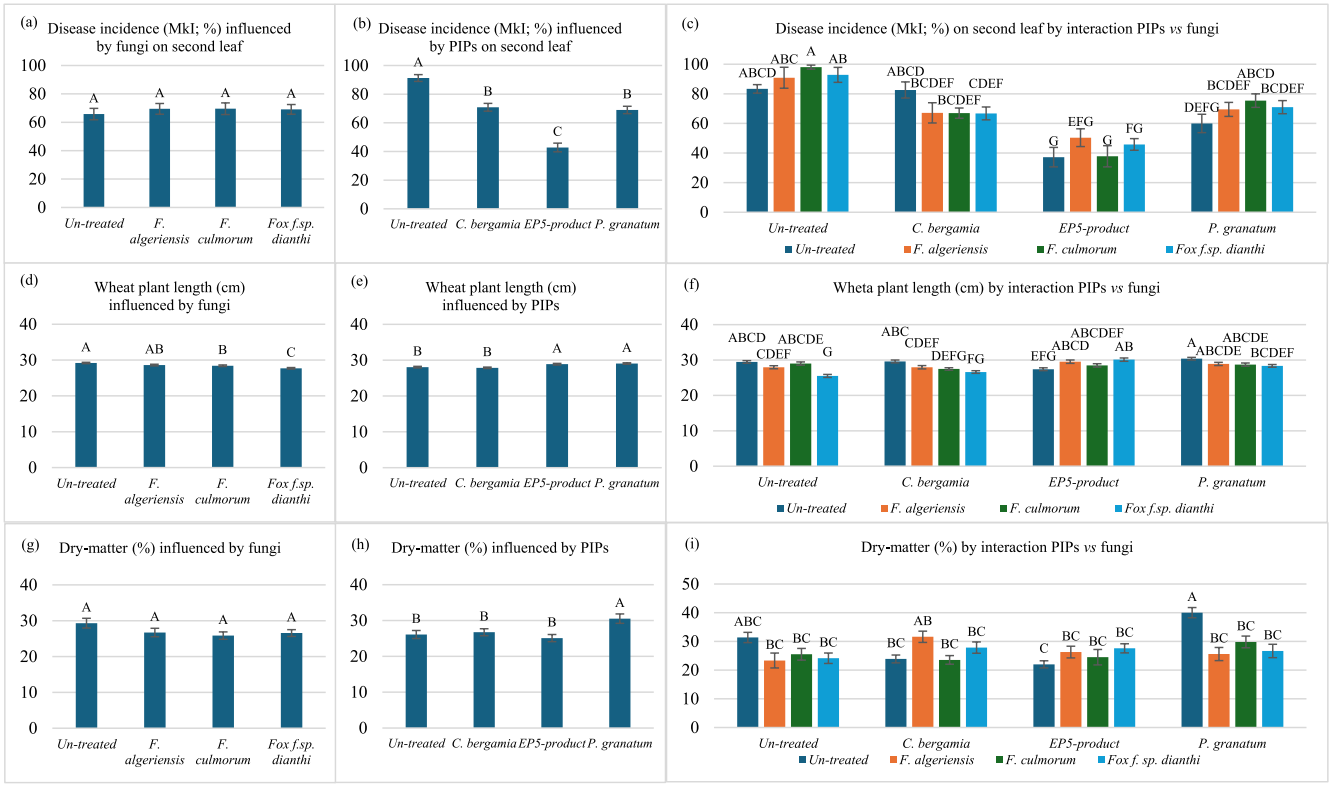


FIGURE 6 | Influence of fungal pathogens, putative inhibiting products (PIPs) treatments, and their combinations on leaf phytosanitary status, plant length, and dry-matter: (a) effect of the presence of the fungal pathogens on the second leaf; (b) effect of the PIPs on the second leaf; c) effect of PIP treatments *versus* fungi on the second leaf; (d) effect of the presence of the fungal pathogens on the plant length; (e) effect of the PIPs on the plant length; (f) effect of the PIP treatments *versus* fungi on the plant length; (g) effect of the presence of the fungal pathogens on the dry-matter; (h) effect of the PIPs on the dry-matter; (i) effect of the PIP treatments *versus* fungi on the dry-matter. One-way and Factorial ANOVA (interaction PIPs *vs* fungi), Tukey Test, $p < 0.01$ were performed; the means with the same capital letters indicate not significant differences.

inoculation effects decreased over time, suggesting limited pathogen survival between experiments, probably due to PIP bio-fumigant activities. Residual PIP activity, especially from EP5-product, continued to influence plant responses, slightly improving germination rate and markedly reducing root and leaf disease symptoms, while enhancing plant length and dry-matter. EP5-product showed the strongest persistent antifungal activity, reflected in reduced disease incidence and low fungal re-isolation rates, despite its initial phyto-toxicity in the first greenhouse experiment. These results indicate that EP5-product is an effective bio-fumigant agent in first greenhouse experiment and, due to its progressive breakdown over time and persistent residual presence, an effective antifungal agent in the second greenhouse experiment.

During the second greenhouse experiment, also bergamot and pomegranate lightly improved all wheat traits, from germination rate to overall phytosanitary and agronomic status, demonstrating lower residual antifungal effects. Nevertheless, their effects suggest potential for sustainable, plant-friendly disease management strategies, although dosage and administration timing adjustments may be need. The results are agreeing with results discussed by Nawaz et al. (2025). Therefore, these results highlight that PIPs exerted long-lasting biological effects beyond the initial treatment period, contributing to the suppression of soil-borne pathogens and improving plant health.

Regarding the metabolic compounds in pomegranate peel and bergamot wastes and their beneficial properties, several studies have focused on extraction methods (water, organic solvent, etc.) (El Sebai et al. 2023), chemical characterization, anti-oxidant activity of human welfare, and antimicrobial properties (Wu et al. 2025). Further investigations will explore the repressiveness of fungal pathogens both in open field and in postharvest conditions.

In conclusion, this study demonstrates that agricultural-waste-derived PIPs and the commercial EP5-product offer promising natural alternatives for managing soil-borne fungal pathogens. *In vitro* assays revealed that antifungal activity is both pathogen-specific and PIP-dependent, with EP5-product showing the most consistent inhibitory performance. Greenhouse experiments confirmed strong bio-fumigant and residual suppressive effects of EP5-product, although high doses caused phyto-toxicity, indicating its suitability mainly for pre-sowing and transplanting applications. Bergamot and pomegranate PIPs, while less potent, were non-phyto-toxic and contributed to improved plant health, suggesting potential for sustainable and plant-friendly disease management. The persistence of antifungal effects across experiments highlights the long-lasting protective influence of these natural products. Overall, the results support further investigation of PIPs and EP5-product as eco-compatible tools for reducing chemical

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pesticide use and enhancing crop protection in both controlled and field conditions.

Author Contributions

Conceptualization, Antonia Carlucci. Methodology, Thomas Conte, Maria Grazia Morea, and Antonia Carlucci. Software, Thomas Conte, Maria Grazia Morea, Angela Libutti and Antonia Carlucci. Validation, Thomas Conte, Maria Grazia Morea, Angela Libutti and Antonia Carlucci. Formal analysis, Antonia Carlucci and Thomas Conte. Investigation, Thomas Conte, Maria Grazia Morea, Angela Libutti and Antonia Carlucci. Data curation, Thomas Conte, Maria Grazia Morea and Antonia Carlucci. Writing – original draft preparation, Thomas Conte, Angela Libutti and Antonia Carlucci. Writing – review and editing, Thomas Conte, Angela Libutti and Antonia Carlucci. Visualization, Thomas Conte, Maria Grazia Morea, Angela Libutti and Antonia Carlucci. Supervision, Antonia Carlucci and Angela Libutti. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

TABLE S1: Sensitivity of the fungal species subjected to inhibitory activity of the putative inhibiting products (PIPs) on artificial medium in *in vitro* conditions. **TABLE S2:** Inhibitory activity of the putative inhibiting products (PIPs) on artificial medium in *in vitro* conditions.

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TABLE S3: Effect of interaction between the fungal species inoculated and the putative inhibiting products (PIPs) by inhibition of fungal growth on Petri dishes in *in vitro* conditions. **TABLE S4:** Statistical effects for the multi-factorial Anova run on the parameter $\Delta\tau$ (prolongation of τ referred to PDA) (hours). **TABLE S5:** Effect of the presence of the inoculated fungal pathogens on the germination of the wheat seeds. **TABLE S6:** Effect played by the putative inhibiting products (PIPs) on the germination of the wheat seeds. **TABLE S7:** Effect of the interaction between the inoculated fungal pathogens and the putative inhibiting products (PIPs) on the germination rates of the wheat seeds. **TABLE S8:** Phyto-toxicity played by the putative inhibiting products (PIPs) on root in presence of the inoculated fungi. **TABLE S9:** Phyto-toxicity played by the putative inhibiting products (PIPs) on root. **TABLE S10:** Effect of the interaction between the inoculated fungal pathogens and the putative inhibiting products (PIPs) on the phyto-toxicity on root. **TABLE S11:** Inoculated fungal pathogens re-isolated from the treated and un-treated root. **TABLE S12:** Effect of the presence of the inoculated fungal pathogens on the putative inhibiting products (PIPs) phyto-toxicity on the second leaf. **TABLE S13:** Putative phyto-toxicity effect of the putative inhibiting products (PIPs) on the second leaf. **TABLE S14:** Effect of the interaction between the inoculated fungal pathogens and the putative inhibiting products (PIPs) on the phyto-toxicity on the second leaf. **TABLE S15:** Effect of the presence of the inoculated fungal pathogens on the putative inhibiting products (PIPs) phyto-toxicity on plant length. **TABLE S16:** Putative phyto-toxicity effect of the putative inhibiting products (PIPs) on plant length. **TABLE S17:** Putative phyto-toxic effect of the putative inhibiting products (PIPs) of the treatments by interaction between fungus and PIP on plant length. **TABLE S18:** Effect of the inoculated fungal pathogens in presence of the putative inhibiting products (PIPs) phyto-toxicity on dry-matter. **TABLE S19:** Putative phyto-toxicity effect of the putative inhibiting products (PIPs) on dry-matter. **TABLE S20:** Effect of the interaction between fungus and the putative inhibiting products (PIPs) on dry-matter. **TABLE S21:** Effect of the probable presence of the inoculated fungal pathogens on the germination of the wheat seeds. **TABLE S22:** Effect played by the residual putative inhibiting products (PIPs) on the germination of the wheat seeds. **TABLE S23:** Effect of the interaction between the inoculated fungal pathogens and the putative inhibiting products (PIPs) on the germination rates of the wheat seeds. **TABLE S24:** Root symptoms caused by the survived inoculated fungi. **TABLE S25:** Root symptoms due to the residual putative inhibiting products (PIPs) activities. **TABLE S26:** Effect of the interaction between the inoculated fungal pathogens and the putative inhibiting products (PIPs) in affecting root health. **TABLE S27:** Survived inoculated fungal pathogens re-isolated from the treated and un-treated root. **TABLE S28:** Effect of the survived fungal pathogens on the second leaf. **TABLE S29:** Effect of the residual putative inhibiting products (PIPs) on the second leaf. **TABLE S30:** Effect of the interaction between the survived inoculated fungi and the residual putative inhibiting products (PIPs) on the second leaf. **TABLE S31:** Effect of the survived fungal pathogens on plant length. **TABLE S32:** Effect of the residual putative inhibiting products (PIPs) on plant length. **TABLE S33:** Effect of the interaction of between the survived fungal pathogens and the residual putative inhibiting products (PIPs) on plant length. **TABLE S34:** Effect of the survived fungal pathogens on dry-matter. **TABLE S35:** Effect of the residual putative inhibiting products (PIPs) on dry-matter. **TABLE S36:** Effect of the interaction between the survived inoculated fungi and the residual putative inhibiting products (PIPs) on dry-matter.

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Objective 3. As production of durum wheat is heavily threatened by the development of weed biotypes exhibiting resistance to single or multiple herbicide mechanisms of action (MoA), such as corn poppy (*Papaver rhoeas* L.), sterile oat (*Avena sterilis* L.), and ryegrass (*Lolium* spp.), water extracts (E) obtained from the powders (P) of bergamot, pomegranate, and EP5 were tested *in vitro* against black-grass (*Alopecurus myosuroides* Huds.), corn poppy, and Italian ryegrass (*Lolium multiflorum* Lam.). After evaluating the effects of these extracts on weed germination and early growth, a second experiment was conducted *in vivo* by applying the powders directly into the soil. This second trial was carried out under greenhouse conditions (*Article 3, submitted to* Frontiers in Agronomy *on 18 June 2026*).

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Allelopathic potential and soil persistence of bergamot (Citrus bergamia L.) and pomegranate (Punica granatum L.) peel powders, as sustainable plant-derived products for the management of resistant weeds associated with durum wheat.

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Scope Statement

The allelopathic potential and weed-suppressive efficacy of water extracts and powders from bergamot, pomegranate fruit peel and the EP5 sustainable commercial product, under both in vitro and in vivo conditions, were evaluated. Two subsequent experiments were carried out. We tested these treatments against major agricultural weeds (Alopecurus myosuroides and Lolium multiflorum) associated with wheat. We also evaluated the persistence of these products over time within the soil against the selected weeds, as well as their potential phytotoxicity on durum wheat germination, early growth, and development. Our findings demonstrated a high in vitro selectivity, with water extracts of bergamot, pomegranate, and EP5 showing strong inhibitory effects on weeds while leaving durum wheat unaffected. Moreover, in the first in vivo experiment, soil application of bergamot, pomegranate, and EP5 powders showed an interesting weed suppression. Interestingly, the second in vivo experiment revealed a shift in behaviour, where the powders began acting as crop biostimulants. The tested powders are highly promising, sustainable, eco-friendly alternatives tailored specifically for pre-emergence applications. This research aligns with the scope of Frontiers in Agronomy on integrated, sustainable, and biological weed control strategies. By bridging the gap between circular economy and modern weed management, our work offers insights for researchers.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Credit Author Statement

Angela Libutti: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Antonia Carlucci**: Investigation, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Maria Grazia Morea**: Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **Thomas Conte**: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing.

Keywords

Agro-industrial by-products, Alopecurus myosuroides Huds., Bergamot, Bio-herbicides, durum wheat, Lolium multiflorum L., pomegranate., Sustainable weed control

Abstract

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Plant-derived products offer a sustainable strategy for weed control while mitigating the ecological, health, and resistance concerns of synthetic chemical herbicides.

This study evaluated the allelopathic potential of bergamot and pomegranate fruit peel powders, alongside the potential weed-suppressive efficacy of the EP5 commercial powder, under in vitro and in vivo conditions.

Powder water extracts were preliminarily tested under laboratory conditions against Alopecurus myosuroides, Lolium multiflorum, and Papaver rhoeas seeds, as well as durum wheat kernels. Subsequently, two greenhouse pot-experiments were carried out to evaluate the powder herbicidal activity against A. myosuroides and L. multiflorum, their phytotoxicity on durum wheat, and persistence over time within the soil. The germination and early development of both weeds and wheat were assessed through a set of germination-related parameters, including the final germination percentage, mean germination time, germination index and seed vigor index, as well as a set of growth-related parameters, such as root, shoot and total seedling lengths, root, shoot and total dry matter content, plant inhibition index and biomass accumulation inhibition.

The in vitro experiment revealed a strong inhibitory effect on weeds without affecting wheat. In the first in vivo experiment, EP5 reduced wheat germination and seed vigor by approximately 50%, while pomegranate reduced wheat dry matter; on weeds, EP5 was the strongest germination inhibitor, all powders reduced root length, and pomegranate maximized overall plant and biomass inhibition. The second in vivo experiment revealed a biostimulatory trend: bergamot and pomegranate accelerated wheat germination, EP5 promoted seedling elongation, and pomegranate enhanced dry matter. Furthermore, seed vigor of weeds increased under all treatments, with bergamot promoting plant length, while pomegranate and EP5 minimized growth inhibition.

The tested powders are promising natural alternatives to chemical herbicides; however, their short-term soil persistence and slight phytotoxicity to wheat suggest that they may be particularly suitable for pre-emergence applications.

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In review

Allelopathic potential and soil persistence of bergamot (*Citrus bergamia* L.) and pomegranate (*Punica granatum* L.) peel powders, as sustainable plant-derived products for the management of resistant weeds associated with durum wheat.

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Keywords: **Agro-industrial by-products, bio-herbicides, sustainable weed control, *Alopecurus myosuroides* Huds., *Lolium multiflorum* L., durum wheat, bergamot, pomegranate.**

Abstract

Plant-derived products offer a sustainable strategy for weed control while mitigating the ecological, health, and resistance concerns of synthetic chemical herbicides.

This study evaluated the allelopathic potential of bergamot and pomegranate fruit peel powders, alongside the potential weed-suppressive efficacy of the EP5 commercial powder, under *in vitro* and *in vivo* conditions.

Powder water extracts were preliminarily tested under laboratory conditions against *Alopecurus myosuroides, Lolium multiflorum,* and *Papaver rhoeas* seeds, as well as durum wheat kernels. Subsequently, two greenhouse pot-experiments were carried out to evaluate the powder herbicidal activity against *A. myosuroides* and *L. multiflorum,* their phytotoxicity on durum wheat, and persistence over time within the soil. The germination and early development of both weeds and wheat were assessed through a set of germination-related parameters, including the final germination percentage, mean germination time, germination index and seed vigor index, as well as a set of growth-related parameters, such as root, shoot and total seedling lengths, root, shoot and total dry matter content, plant inhibition index and biomass accumulation inhibition.

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The tested powders are promising natural alternatives to chemical herbicides; however, their short-term soil persistence and slight phytotoxicity to wheat suggest that they may be particularly suitable for pre-emergence applications.

In review

1. Introduction

Global food security faces a critical challenge as the world population is projected to reach 10 billion by 2050, triggering a sharp increase in food demand. Reducing food loss and waste is widely recognized as a key strategy to enhance food system efficiency, improve nutrition, and promote environmental sustainability (FAO, 2019). Concurrently, addressing food security requires minimizing production-stage losses caused by abiotic and biotic environmental factors, which impair crop performance and reduce yields (Oerke, 2006). According to FAO (2019), approximately 20-40% of global crop production is lost due to pests, including pathogens, insects and weeds (Savary et al., 2019). These biotic stressors significantly impact major food crops, especially wheat, rice, maize and potato, which are the three main cereals and the leading tuber produced globally in 2023 (FAO, 2024). Specifically for wheat, potential losses attributed to weeds are estimated at 23%; however, crop protection measures reduce these losses to 8%. Overall, weeds account for the highest potential loss (34%), with animal pests and pathogens being less harmful (18% and 16% yield losses, respectively) (Oerke, 2006). Weeds can exert both positive and negative effects on agro-ecosystems. They can serve as a food source for earthworms, pollinators, birds, mammals and animals that act as natural pest control agents (Petit et al., 2010). Conversely, the synchronized life cycle of crops and weeds makes the latter highly competitive, resulting in intense competition for essential resources such as light, water, nutrients and soil space. The dynamic of weed-crop competition changes throughout the crop development stages. Weeds exert their greatest detrimental impact during the initial stages, primarily due to the slower early crop establishment. This difference in initial growth rate affects both nutrient acquisition and light interception; indeed, it has been reported that crop shading of 44-56% by weed canopies results in 19% to 25% yield reduction (Korav et al., 2018).

In Italy, wheat is one of the main cultivated crops. In 2023, production reached 6.885 million tonnes, comprising 3.804 million tons of durum wheat (*Triticum durum* Desf.) and 3.081 million tons of common wheat (*Triticum aestivum* L.). Durum wheat cultivation is mainly concentrated in Southern Italy, which accounts for 80% of the national cultivated area and 71% of the national production; the Apulia region alone represents 29% of the national cultivated area and 24% of the national production (ISMEA, 2024). However, Italian wheat production is heavily threatened by the development of weed biotypes exhibiting resistance to single or multiple herbicide mechanisms of action. According to Bayer (2026), this phenomenon is mainly documented in species such as poppy (*Papaver* spp.), mustard (*Sinapis* spp.), oats (*Avena* spp.), foxtail (*Alopecurus* spp.), phalaris (*Phalaris* spp.) and ryegrass (*Lolium* spp.), with particularly high incidence in the Apulia region. Among these, poppy (*Papaver rhoeas* L.), oats (*Avena sterilis* L.) and ryegrass (*Lolium* spp.) are the most concerning due to the widespread resistance to a wide range of herbicides, such as acetolactate synthase (ALS) inhibitors and Acetyl-CoA carboxylase (ACCase) inhibitors.

The development of resistant weed populations is closely linked to the extensive herbicide use (Cullen et al., 2019; Dolatabadi et al., 2021; Montull and Torra, 2023), which has progressively supplanted alternative weed management practices over recent decades (Nandula, 2019). Although effective in weed control, herbicides have negative effects on both human and environmental health; their overuse has been linked to an increased incidence of human pathologies, alongside the accumulation of toxic residues in water bodies, soils, and biological tissues. Furthermore, these compounds inadvertently harm non-target and beneficial organisms (Cavalier et al., 2023; Wilms et al. 2020). Consequently, a drastic reduction in synthetic pesticide application is needed to generate public health, environmental and economic benefits (Pimentel and Burgess, 2014), aligned with the directives of the European Common Agricultural Policy. Notably, a key ambition of the "Farm to Fork Strategy", a central pillar of the "European Green Deal", is to achieve a 50% reduction in the chemical pesticide use across the EU by 2030, thereby fostering a more sustainable agricultural model.

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Plant-derived products offer suitable and eco-friendly alternatives for weed management. Numerous plant species are currently being investigated for their bioherbicidal properties; specifically, extracts and essential oils from eucalyptus (*Eucalyptus globulus* Labill.) and acacia (*Acacia dealbata* Link), spearmint (*Mentha spicata* L.), cinnamon (*Cinnamomum zeylanicum* Blume), and oregano (*Origanum vulgare* L.) strongly suppress both seed germination and seedling vigor (Kanatas, 2020; Kordali et al., 2022; Verdeguer et al., 2020). Additionally, cover and cash crops, such as sorghum (*Sorghum bicolor* L. Moench), rye (*Secale cereale* L.), and sunflower (*Helianthus annuus* L.), effectively control weeds (Jabran et al., 2015; Scavo and Mauromicale, 2021). Recent studies highlight the herbicidal potential of bergamot (*Citrus bergamia* Risso) and pomegranate (*Punica granatum* L.). Bergamot essential oil reduces weed germination under both *in vitro* and *in vivo* conditions, while its extract is effective as a post-emergence bioherbicide (Bozhuyuk, 2020; Tienebo et al., 2025). Similarly, pomegranate peel essential oil inhibits the germination of a wide range of weeds (Taha and Othman, 2025). These herbicidal effects are driven by complex mixtures of secondary metabolites, including alkaloids, phenols, and terpenoids, which suppress weed germination and growth through allelopathic interactions (Jabran et al., 2015; Satyanarayana Murthy et al., 2025; Scavo et al., 2019). Unlike synthetic herbicides that typically target a single metabolic site, these natural compounds often possess multiple modes of action, reducing the probability of weed resistance evolution (Duke et al., 2024; Verdeguer et al., 2020). Despite the phytotoxicity of plant secondary metabolites, validating their efficacy against highly competitive weeds that jeopardize crop production is essential. Within winter cereal systems, species like *Alopecurus myosuroides* Huds., *Papaver rhoeas* L. and *Lolium multiflorum* L. are particularly suitable model species for evaluating novel plant-based solutions due to their rapid early establishment, synchronized germination with the crop cycle, as well as the capacity to evolve cross-resistance to conventional herbicides. However, the practical application of essential oils and plant extracts is frequently constrained by high volatility, which limits their persistence in the soil. This necessitates alternative strategies that facilitate a more gradual release of allelochemicals, thereby extending their inhibitory activity over time. In this regard, soil incorporation of plant by-products emerges as a promising approach to ensure direct contact between plant secondary metabolites and the weed seedbank. Nevertheless, such persistence must not be excessively prolonged, thereby preventing potential phytotoxic injury to subsequent cash crops. Therefore, a rigorous phytotoxicity assessment is crucial to ensure that such persistence targets weeds without harming the main crop.

In this context, and driven by the need for sustainable weed control in winter-cereal crops, particularly in the Apulia region (Southern Italy) where weed resistance is widespread, this study focuses on the herbicidal potential of powders from bergamot and pomegranate fruit processing by-products applied as allelopathic soil amendments. To expand the range of sustainable strategies, the study also evaluates EP5, a commercial mineral powder containing 5% citric acid. Although developed as a resistance inducer against fungal pathogens, its organic acid matrix suggests a potential herbicidal activity. Notably, Alpi et al. (2025) demonstrated that citric acid provides effective pre-emergence weed control by inhibiting germination and shoot emergence, while remaining selective and safe for soft wheat. Therefore, the aims of this study were to evaluate: 1) the allelopathic inhibitory potential of dried peel powders from bergamot (*Citrus bergamia* L.) and pomegranate (*Punica granatum* L.), alongside the herbicidal potential of the EP5 commercial powder, against three major wheat-associated weeds, namely *Alopecurus myosuroides* Huds., *Papaver rhoeas* L. and *Lolium multiflorum* L.; 2) the persistence of bergamot, pomegranate and EP5 powders over time within the soil against the selected weeds; 3) the potential phytotoxicity of bergamot, pomegranate and EP5 powders on wheat germination, early growth and development.

2. Materials and methods

2.1 Powder preparation and target weed species

For the experiment, powders obtained from bergamot (*Citrus x bergamia* (Risso) Risso & Poit) and pomegranate (*Punica granatum* L.) dried fruit peels were used. Pomegranate peels were sourced as by-products of juice extraction from a farm located in the agricultural area of Foggia district (Apulia region, Southern Italy); bergamot peels were sourced as essential-oil extraction by-products from a farm located in the agricultural area of Gioia Tauro (Calabria region, Southern Italy). Following the protocol of Morea et al. (2025), the plant matrices were processed by gently washing the peels, sectioning them into small fragments, and air-drying them under room conditions until an approximate weight loss of 50% was achieved. The semi-dehydrated peels were subsequently oven-dried at 30°C (FN 500 Sterilizer, Nuve, Ankara TR) until a constant weight was reached. The resulting material was finely ground by an electric mill equipped with a fine-mesh screen (Geomil-50P) at 600 µm and stored in opaque plastic containers under dark conditions. In addition to bergamot and pomegranate powders, the EP5 commercial powder, a fungal resistance inducer approved for organic agriculture, was tested. The formulation of EP5 consisted of a mineral fraction, including iron (10%), zinc (6%), and manganese sulphate (6.5%), combined with bentonite silicates (30%), and 5% citric acid as the organic acid component (Phenom Biotech s.r.l., Milan Italy).

To evaluate the potential allelopathic efficacy of the pomegranate and bergamot powders, alongside the potential herbicidal activity of EP5 powder, three weed species were selected: *Alopecurus myosuroides* Huds., *Papaver rhoeas* L. and *Lolium multiflorum* L. These species were chosen due to their negative impact on wheat yield in the Foggia district and their widespread and increasing resistance to synthetic herbicides. To guarantee experimental uniformity and high germination quality, the seeds were sourced from specialized commercial seed suppliers.

2.2 *In vitro* allelopathic evaluation

A preliminary *in vitro* bioassay was carried out at the Laboratory of Agronomy of the University of Foggia (41°27′ N - 15°33′ E, 76 m a.s.l., Apulia region, Southern Italy), to assess the allelopathic inhibitory potential of water extract of bergamot and pomegranate peel powders, as well as the herbicidal potential of water extract from EP5 commercial powder on the kernels of durum wheat (*Triticum durum* Desf., "*RGT Voilur*" cultivar, Semetica, Bologna, Italy) and the seeds of the three selected weeds (*A. myosuroides*, *P. rhoeas* and *L. multiflorum*), prior to the pot-experiments. According to Scavo et al. (2020), the water extracts were prepared by soaking the powders of bergamot and pomegranate in sterilized distilled water (1:10, w/v) into Erlenmeyer flasks with beaded rim and incubating in the dark for 72 hours at 25.0°C. The mixtures were then centrifuged (Beckman Allegra 25R, Beckman Coulter Inc., Brea, CA, USA) at 20,000 g for 15 min at 18°C to separate the solid and liquid fractions and the supernatant was filtered sequentially through cheesecloth and filter papers (Whatman Inc., Buckinghamshire, UK) of decreasing pore size, from 1 to 0.45 until 0.22 µm, to remove microorganisms and ensure sterility for the *in vitro* bioassay. The same procedure was applied to EP5 commercial powder. The sterile extracts of bergamot, pomegranate and EP5 were stored in sterile bottles at 5.0°C until use.

For the experiment, durum wheat kernels and the seeds of *A. myosuroides*, *P. rhoeas* and *L. multiflorum* were surface-sterilized to prevent fungal contamination. Based on a modified protocol by Fisher et al. (1992), kernels and seeds were sequentially immersed in 70% ethanol, 2% sodium hypochlorite and 70% ethanol. The exposure time to the sterilizing solutions was 15 s for *A. myosuroides* and *P. rhoeas*, and 30 s for *L. multiflorum* and *T. durum*., so as to avoid embryo devitalization. Subsequently, the sterilized kernels and seeds were dried on a sterile filter paper disc under a laminar flow cabinet. Following the method described by Scavo et al. (2020), twenty-five

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kernels of wheat or seeds per species were placed in 90 mm Petri dishes containing two layers of sterile Whatman papers No. 2, moistened with 5 mL of each water extract. Sterilized distilled water was used as the control treatment (E_0). The Petri dishes plates were subsequently sealed with Parafilm® to prevent extract evaporation and incubated for 14 days under natural photoperiod and room temperature. The experiment followed a completely randomized factorial design (CRD) with four species (*T. durum*., W; *A. myosuroides*, Alo; *P. rhoeas*, Pap; *L. multiflorum*, Lol) x four extract types (bergamot, E_B; pomegranate, E_P; EP5, E_{EP5}; distilled water or control, E_0) x four replications. The combination of the two experimental factors, Species (S) and Extract (E), resulted in sixteen experimental treatments reported in Supplementary Table 1. The number of germinated seeds was counted daily with germination defined as primary root protrusion ≥ 2.00 mm.

After 14 days, the number of normal seedlings, defined as those displaying both roots and shoot, was recorded and a set of growth-related parameters were determined. Specifically, root length (RL, mm) was measured from the start to the root tip, shoot length (SL, mm) was measured from the base to the shoot apex, and total length (TL, mm) was determined as the sum of RL and SL. After weighing the roots and the shoot of each seedling to obtain their respective fresh weights (RFW and SFW, g, respectively), as well as the total fresh weight (TFW, g) as the sum of RFW and SFW, roots and shoot were oven-dried at 60 °C for about 48 hours, until a constant weight was achieved. Subsequently, the dry weight of roots (RDW, g) and shoot (SDW, g), and total dry weight (TDW, g) calculated as sum of RDW and SDW, were determined.

To evaluate the impact of water extracts on seed germination (Božena, 2023), a set of germination-related parameters was determined, including the final germination percentage (FGP, %), the germination index (GI) and the mean germination time (MGT, days). Specifically, the final germination percentage (FGP) was calculated according to the following formula:

$$FGP = \frac{\Sigma n}{TN} x\ 100 \quad [1]$$

where Σn is the total number of germinated seeds; TN is the total number of seeds left to germinate. The mean germination time (MGT) was calculated according to Ellis and Roberts (1981) using the following formula:

$$\mathrm{MGT} = \frac{\sum(\mathrm{G_I} \times \mathrm{T_i})}{\mathrm{N}} \quad [2]$$

where G_i is the number of germinated seeds on day *i*, T_i is the day number from the start of the experiment, and N is the total number of seeds germinated at the end of the experiment.

The germination index (GI) was calculated according to Maguire (1962) using the following formula:

$$\mathrm{GI} = \sum \frac{\mathrm{G_i}}{\mathrm{T_i}} \quad [3]$$

where G_i is the number of germinated seeds on day i, and T_i is the day number from the start of the experiment until the day of the specific count.

To evaluate the impact of water extracts on seedling growth (Božena, 2023), a set of growth-related parameters was determined, including the root (RDM, % fw), shoot (SDM, % fw), and total dry matter content (TDM, % fw), the seed vigor index (SVI), the plant inhibition index (PII, %) and the biomass accumulation inhibition (BAI, %). The dry matter content for each seedling component was calculated according to the following formula:

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$$DM_i(\%\ fw) = \frac{DW_i\ (g)}{FW_i\ (g)} \quad [4]$$

where DW is the dry weight, FW is the fresh weight and i refers to the root (R), shoot (S) or total (T).

The seed vigor index (SVI) was calculated according to Al-Jibouri et al. (1958) using the following formula:

$$SVI = FGP\ (\%)\ x\ Total\ seedling\ length\ (cm) \quad [5]$$

The plant inhibition index (PII) was calculated according to Attard et al. (2015) using the following formula:

$$PII = \frac{Control\ seedling\ length\ (cm) - Treated\ seedling\ length\ (cm)}{Control\ seedling\ length\ (cm)}\ x\ 100 \quad [6]$$

The biomass accumulation inhibition (BAI) was calculated according to Ismil et al. (2019) using the following formula:

$$BAI = \frac{Control\ seedling\ fresh\ weight\ (g) - Treated\ seedling\ fresh\ weight\ (g)}{Control\ seedling\ fresh\ weight\ (g)}\ x\ 100 \quad [7]$$

In addition, data regarding the number of the roots produced by each wheat kernel and weed seed, as well as their individual length were collected to determine the average root number (AVG_{RN}) and the average length of individual root (AVG_{RL}) per emerged seedling and used to evaluate the allelopathic effects of the water extracts.

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2.3 *In vivo* allelopathic evaluation

Based on the results of the preliminary in vitro experiment, *P. rhoeas* was excluded from further investigation because its seeds failed to germinate even in the control treatment. Therefore, only *A. myosuroides* and *L. multiflorum* were considered for the *in vivo* evaluation.

Specifically, two subsequent pot-experiments were carried out at the greenhouse of the University of Foggia (41°27′ N - 15°33′ E, 76 m a.s.l.), Apulia region, Southern Italy, under natural conditions of light and day/night temperature, from February to May 2024. Durum wheat kernels (*T. durum* Desf., cv. "RGT Voilur"; Semetica, Bologna, Italy) were sown in 1.5-L cylindrical plastic pots filled with a commercial substrate, under either weedy (*A. myosuroides* and *L. multiflorum*) or weed-free conditions, and under either treated (bergamot, pomegranate and EP5 powders) or un-treated soil conditions.

2.3.1 First *in vivo* experiment

For the first experiment, pots were prepared by placing two layers of non-woven fabric at the bottom to prevent soil loss and filling them to approximately 2/3 of their height (6 cm) with "Universal Gaia" substrate (Agrochimica S.p.A., Bolzano, Italy). 30 seeds of each selected weed species and 10.0 g of each plant powder were placed on the soil surface of the weedy and powder-treated pots, respectively, and covered with 1.0 cm of soil layer. Each pot was then watered and covered with a plastic film. After 72 hours, 30 durum wheat kernels were sown at an equal distance from one another in both the weedy and weed-free pots, as well as in both the powder treated and un-treated pots, and covered with an additional 1.0 cm soil. Weed-free and un-treated pots served as controls. The experiment was arranged according to a completely randomized factorial design (CRD) with three weed species (*A. myosuroides*, W_{Alo}; *L. multiflorum*, W_{Lol}; no weed or weed-free control, W_0) x four powder types (bergamot, P_B; pomegranate, P_P; EP5, P_{EP5}; no powder or un-treated control, P_0) x sixteen replicates,

for a total of 192 experimental units. The combination of the two experimental factors, weed (W) and powder (P), resulted in the twelve experimental treatments reported in Supplementary Table 2.

The number of germinations for both wheat kernels and weed seeds was recorded every 48 hours over a 45-day period. As in the *in vitro* experiment, daily germination data were used to determine the germination-related parameters, namely the final germination percentage (FGP, %), mean germination time (MGT, days) and germination index (GI), according to the formulas described in the Section 2.2.

After 45 days, the plants were gently removed from the pots and the roots were carefully washed to remove all soil residues. After length and fresh weight measurements of plant roots and shoot, the two plant components were oven-dried at 60 °C for about 48 hours, until a constant weight was achieved and their dry weights were recorded. As in the *in vitro* experiment, the growth-related parameters, namely, root, shoot and total lengths (RL, SL and TL, cm, respectively) were measured, and the root, shoot and total dry matter content (RDM, SDM and TDM, % fw, respectively), the seed vigor index (SVI), the plant inhibition index (PII, %) and the biomass accumulation inhibition (BAI, %) were determined using the protocols and equations reported in the Section 2.2.

2.3.2 Second *in vivo* experiment

The second experiment was carried out two weeks after the termination of the first one, by re-sowing the same pots with 30 kernels of durum wheat and 30 seeds of each weed species (*A. myosuroides* and *L. multiflorum*), across all twelve previous treatment combinations. As in the first experiment, weed-free and un-treated pots served as controls. Consistent with the first trial, the experiment followed a completely randomized factorial design (CRD) with three weed species (*A. myosuroides*, W_{Alo}; *L. multiflorum*, W_{Lol}; no weed or weed-free control, W_0) x four powder types (bergamot, P_B; pomegranate, P_P; EP5; no powder or un-treated control, P_0) x sixteen replicates, for a total of 192 experimental units. The combination of the two experimental factors, weed (W) and powder (P), resulted in twelve experimental treatments that were identical to those of the first pot-trial (Supplementary Table 2). Germination of wheat and weeds was monitored every 48 hours for 45 days to calculate the germination-related parameters, namely the final germination percentage (FGP, %), mean germination time (MGT, days) and germination index (GI). At the end of this period, plants were harvested and roots were carefully cleaned to measure the growth-related parameters namely, root, shoot and total lengths (RL, SL and TL, cm, respectively) were measured, and the root, shoot and total dry matter content (RDM, SDM and TDM, % fw, respectively), the seed vigor index (SVI), the plant inhibition index (PII, %) and the biomass accumulation inhibition (BAI, %), following the protocols and the equations described in the Section 2.2.

2.4 Statistical analyses

All experimental data were first checked for normality and homogeneity of variances using the Shapiro–Wilk ($p \leq 0.05$) and Bartlett ($p \leq 0.05$) tests, respectively, and then tested for differences using analysis of variance (ANOVA). The statistical significance of the differences between the means was analyzed using Tukey's honest significant difference (HSD) post hoc test at the 5% probability level. Specifically, data from the *in vitro* experiment were analyzed by two-way ANOVA to examine the effect of the factors: Species (three levels, W; Alo and Lol) and Extract (four levels, E_0, E_B, E_P, E_{EP5}), as well as their interactions (S x E). Data from the first and second *in vivo* experiments were analyzed by two-way ANOVA to examine the effects of Weed (W: three levels, W_0, W_{Alo}, W_{Lol}), Powder (P: four levels, P_0, P_B, P_P, P_{EP5}), as well as their interactions (W x P). Particularly, for the *in vivo* experiments, the datasets related to wheat and the datasets related to weeds were analyzed independently. This distinct approach was implemented to separately evaluate the potential phytotoxic effects of the powders on the germination and early development of wheat and their actual

allelopathic and bioherbicidal efficacy against the target weed species. All statistical analyses were performed using the JMP software package, version 16.2 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 In vitro allelopathic evaluation

As detailed in Section 2.3., *P. rhoeas* seeds failed to germinate also in the control plates; consequently, no data were collected and statistically analyzed for this weed species, and no results are reported herein.

Germination-related parameters observed on wheat kernels, *A. myosuroides* and *L. multiflorum* seeds, treated with bergamot, pomegranate and EP5 water extracts, are reported in **Table 1**. Significant effects ($p < 0.001$) were observed for both the two experimental factors, Species (S) and Extract (E), as well as their interaction (S x E), across all measured parameters, namely final germination percentage (FGP), mean germination time (MGT), germination index (GI) and seed vigor index (SVI). Wheat kernels displayed the highest FGP, GI and SVI values along with the lowest MGT, demonstrating a higher tolerance to the tested water extracts in comparison to the two weeds, *A. myosuroides* and *L. multiflorum* that, on the contrary, exhibited a markedly higher susceptibility across all the considered parameters. Among the water extracts, EP5 (E_{EP5}) exhibited the highest inhibitory activity, reducing FGP by 98% and GI by 100% compared to the control (E_0). It was followed by bergamot extract (E_B) that accounted for 90% and 96% FGP and GI reductions, respectively, compared to E_0. Pomegranate (E_P) did not differ from either E_B or E_{EP5}, showing 82% and 89% lower FGP and GI values than the in-treated control (E_0). All water extracts led to a notable increase in MGT compared to E_0, indicating a germination delay that was highest under the E_{EP5}, followed by E_B and E_P, which didn't differ from each other. Furthermore, the three extracts caused a drastic reduction in SVI compared to E_0; specifically, E_{EP5} again exhibited the strongest inhibitory impact, reducing this parameter nearly to zero, while E_B and E_P resulted, on average, in a 95% reduction. Notably, E_{EP5} completely suppressed the germination of both *A. myosuroides* and *L. multiflorum* seeds, bringing all germination-related parameters to zero. A similar complete inhibition was induced in *A. myosuroides* by both E_B and E_P. Moreover, E_B and E_P also caused a drastic reduction in *L. multiflorum* germination, decreasing FGP, GI and SVI values by 90%, 95% and 96%, respectively, alongside a substantial increase in MGT (150% and 200% for E_B and E_P, respectively), compared to E_0.

Regarding the growth-related parameters of the emerged seedlings, namely root, shoot and total lengths (RL, SL and TL, respectively) (**Figure 1**), significant effects ($p < 0.001$) were observed for both factors, Species (S) and Extract (E). Wheat always exhibited higher total, root and shoot length values than *A. myosuroides* (**Figure 1 A, B and C**). Moreover, all tested water extracts impaired seedling development (**Figure 1 D**), primarily affecting the root system (**Figure 1 E**). Specifically, RL was drastically reduced by the E_B, E_P and E_{EP5}, which showed no significant differences among them, averaging 90% shorter roots than the un-treated control (E_0). Although SL was always consistently reduced across all treatments (**Figure 1 F**), the most pronounced inhibition occurred under E_{EP5} that led to 99% shorter shoot compared to E_0. In contrast, E_B and E_P determined lower reduction in shoot lengths reductions (40% and 43%, respectively) compared to E_0, while not statistically differing from each other. The interaction S x E significantly ($p < 0.001$) influenced only root elongation (**Supplementary Figure 1**). For all emerged seedling, namely wheat under the E_B, E_P and E_{EP5} treatments and *L. multiflorum* under the E_B and E_P treatments, RL was invariably and significantly reduced compared to their respective controls.

Turning to the root, shoot and total dry matter content (RDM, SDM and TDM, respectively) of the emerged seedlings (**Supplementary Figure 2**), both factors, specie (S) and extract (E), exerted a

significant effect ($p < 0.001$ for both factors, except for the effect of E on RDM, which was significant at $p < 0.05$). In contrast, the interaction E x S showed no significant influence. Among the considered species, the lowest TDM and SDM values were recorded in *A. myosuroides* and *L. multiflorum* seedlings (**Supplementary Figure 2A and B**), which did not differ significantly from each other, exhibiting an average reduction of 78% and 77%, for TDM and SDM respectively, compared to wheat. In contrast, RDM was significantly reduced only in *A. myosuroides*, accounting for a 97% lower value compared to both wheat and *L. multiflorum* (**Supplementary Figure 2C**). Among the tested extracts, only E_{EPS} significantly reduced the RDM, SDM, and TDM values (**Supplementary Figure 2D, E and F**) compared to the un-treated control (E_0).

The results of the inhibitory pressure exerted by the extracts on the post-germinative development of the emerged seedlings, expressed as the plant inhibition index (PII) for length and the biomass accumulation inhibition (BAI) for weight, are reported in **Supplementary Table 3**. Regarding PII, both factors, Species (S) and Extract (E), as well as their interaction (S x E), exerted significant effects ($p < 0.001$). Among the species, *A. myosuroides* exhibited the highest sensitivity, showing a PII equal to 75.0%, followed by *L. multiflorum* (PII = 63.8%) and wheat (PII = 56.0 %), which didn't differ from each other. Concerning the extracts, E_{EP5} was the most inhibiting, with a PII value close to 100%, closely followed by E_B and E_P, which averaged a PII value equal to 80%. Furthermore, the extract-induced inhibition differed based on the species. While E_{EP5} completely suppressed the growth of both wheat and weed seedlings (PII values equal to 100% for the two weeds and 99 for the wheat), E_P and E_B showed a more pronounced inhibitory effect only on the two weeds than on wheat. In contrast, for BAI (**Supplementary Table 3**), only the factor Extract (E) showed a significant effect ($p < 0.001$). Compared to the un-treated control (E_0), the three extracts displayed a comparable inhibitory capacity, resulting in an average BAI value of 98%.

Further analysis of the root system showed that the average root number (AVG_{RN}) per emerged seedling (**Supplementary Table 4**) was significantly ($p < 0.001$) affected by both factors, Species (S) and Extract (E), as well as by their interaction (S x E). Among the species, the two weeds showed a reduced AVG_{RN} accounting, on average, for a 74% lower value than wheat. Regarding the extracts, E_P and E_{EP5} reduced the AVG_{RN} value (on average of 75%) to a similar extent compared to both the un-treated control (E_0) and E_B, which didn't differ from each other. The average length of individual root (AVG_{RL}) per emerged seedling (**Supplementary Table 4**) was significantly influenced ($p < 0.001$) only by the factor Extract (E), with E_B, E_P and E_{EP5} exerting a comparable inhibitory effect and resulting in an 86% lower AVG_{RL} compared to the untreated control (E_0).

3.2 *In vivo* allelopathic evaluation: first experiment

3.2.1 Wheat

In the first greenhouse experiment, the two weed species, *A. myosuroides* and *L. multiflorum*, had no impact on wheat germination, indicating the absence of early competitive effects, as showed by the final germination percentage (FGP) of kernels (**Table 2**) that didn't vary in presence or absence of weeds, averaging approximately 80%. Similarly, mean germination time (MGT), germination index (GI), and seed vigor index (SVI) of wheat kernels remained completely unaffected by the factor Weed (W) (**Table 2**). Conversely, the factor powder (P) showed a significant effect ($p < 0.001$) on all germination-related parameters of wheat. Specifically, P_{EP5} exhibited a strong inhibitory activity, almost halving the FGP in comparison to the un-treated control (P_0), as well as P_B and P_P. Moreover, P_{EP5} considerably increased of about 30% the MGT and reduced GI by 21%, compared to P_0. It was followed by P_B and P_P, which did not differ from each other, averaging a 30% higher MGT and a 25% lower GI. P_{EP5} also reduced the SVI, halving its value compared to both the untreated control (P_0) and the two treatments, P_B, and P_P.

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The growth-related parameters, namely root, shoot and total length (RL, SL and TL, respectively) were significantly ($p < 0.001$) affected by both weed competition (W) and inhibitory effect of powders (P) (**Table 3**). Between the two weeds, *L. multiflorum* (Lol) was the only one to affected all the three length components of wheat plants, reducing RL, SL and TL by 7%, 5% and 6%, respectively, compared to the weed-free control (W_0) and *A. myosuroides*. P_{EP5} was the treatment that more consistently affected wheat RL, SL and TL. Specifically, among the three length components, RL was more strongly inhibited than SL and TL, showing a 21% reduction compared to the 16% and 18% reductions observed in shoot and total length, respectively. The P x W interaction also significantly ($p < 0.001$) influenced the three wheat length components (**Table 3**). Specifically, wheat plants treated with P_B showed a 14% lower RL in the presence of both weeds (W_{Alo}-P_B and W_{Lol}-P_B that didn't differ from each other) than under the weed-free control (W_0-P_B). For wheat plants treated with P_P a 12% reduction occurred in the presence of *L. multiflorum* (W_{Lol}-P_P) compared to both the weed-free control (W_0-P_P) and the presence of *A. myosuroides* (W_{Alo}-P_P), which shared no significant differences. Conversely, P_{EP5} treatment reduced RL by 24%, on average, across all weed conditions (W_0-P_{EP5}, W_{Alo}-P_{EP5} and W_{Lol}-P_{EP5}) compared to the weed-free and un-treated control (W_0-P_0). Furthermore, in the presence of *L. multiflorum*, wheat plants grown on both un-treated (W_{Lol}-P_0) and P_B treated soil (W_{Lol}-P_B) reduced their SL compared to their respective weed-free controls (W_0-P_0 and W_0-P_B). In the presence of *A. myosuroides* wheat plants exhibited a similar decrease in SL only under P_P treatment (W_{Alo}-P_P) compared to both the un-treated control (W_{Alo}-P_0) and P_B treatment (W_{Alo}-P_B). Under both *A. myosuroides* and *L. multiflorum* infestation, soil treatment with P_{EP5} (W_{Alo}-P_{EP5} and W_{Lol}-P_{EP5}, respectively) resulted in about 10% higher SL values than those observed in weed-free soil (W_0-P_{EP5}). Regarding the effects of W x P interaction on the TL of wheat plants, the results aligned with the trends observed for RL and SL (**Table 3**). Due to the reduction in shoot and/or root growth, wheat plants treated with P_B exhibited a decrease in TL in presence of both weeds (W_{Alo}-P_B, W_{Lol}-P_B); those treated with P_P reduced their TL value under *L. multiflorum* infestation (W_{Lol}-P_P); finally, wheat plants treated with P_{EP5} shower higher TL values when grown in presence of both *A. myosuroides* and *L. multiflorum* (W_{Alo}-P_{EP5} and W_{Lol}-P_{EP5}) than their respective weed-free control (W_0-P_B, W_0-P_P and W_0-P_{EP5}). Regarding the root, shoot and total dry matter content (RDM, SDM, and TDM, respectively) (**Table 3**), the factor Weed (W) didn't exhibit a significant influence, in contrast to the factor Powder (P), which was highly significant ($p < 0.001$); the interaction W × P only influenced the SDM and TDM values. Among the powders, P_P led to a significant decrease in SDM (-20%) and TDM (-18%) compared to the untreated control (P_0), while reducing by 33% RDM compared to P_{EP5}. The latter didn't affect any of the three dry matter components, always showing RDM, SDM and TDM values comparable to those of the control (P_0). Furthermore, in interaction with weeds, P_{EP5} significantly reduced the SDM (-29%) in the presence of both weeds (W_{Alo}-P_{EP5} and W_{Lol}-P_{EP5}) and the TDM (-25%) in the presence of *L. multiflorum* (W_{Lol}-P_{EP5}) compared to the weed-free control (W_0-P_{EP5}).

Finally, considering the Plant Inhibition Index (PII) and the Biomass Accumulation Inhibition (BAI), which quantify the inhibitory pressure of the experimental treatments on wheat plants elongation and fresh weight, respectively, no significant effects were observed for the factor Weed (W) or the interaction W x P. Conversely, the factor Powder (P) was found to be highly influential ($p < 0.001$) (**Supplementary Figure 3**). Specifically, P_{EP5} proved to be the most inhibitory powder treatment, showing a high, positive PII value (21%), followed by P_P (PII = 8%). whereas P_B promoted plant elongation. yielding a negative PII value (-0.9%). A different trend was observed for the BAI; in this case, P_P was the only powder treatment to significantly reduce fresh weight in wheat plants, accounting for the highest value (49%), unlike the two treatments, P_{EP5} and P_B, which similarly to the control (P0), promoted wheat plant growth, averaging a negative BAI value equal to -56%.

3.2.2 Weeds

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Supplementary Table 5 shows the germination-related parameters determined on the weeds in the first *in vivo* experiment. Significant differences ($p < 0.001$) were observed between the two weed species, with *L. multiflorum* exhibiting higher germination potential compared to *A. myosuroides.* Specifically, an almost double FGP, GI and SVI were observed on *L. multiflorum*, although the MGT didn't differ significantly between the two species, averaging approximately 20 days. A significant effect ($p < 0.001$) of the factor Powder (P) was also detected (**Supplementary Table 5**), with P_{EP5} emerging as the most effective inhibitor, which reduced the FGP and the GI to the lowest values, followed by P_B and P_P that didn't differ from each other. The powder treatments also delayed the emergence of the weeds, as evidenced by the MGT, which increased passing from 14 days of the un-treated control (P_0) to over 20 days in all powder treatments, peaking at 24 days under P_{EP5}. Similarly, the GI dropped from the highest value in the un-treated control (P_0) to half that value under P_B and P_P, reaching its minimum under P_{EP5}, thus indicating that the tested powders drastically reduced the speed and efficiency of weed germination. Moreover, despite the drastic reduction in FGP, the SVI under P_{EP5} remained statistically comparable to that of the control, likely due to the high performance of the few surviving individuals; in contrast, it drastically decreased under P_B and P_P. The W x P interaction was statistically significant ($p < 0.001$) only for FGP and GI (Supplementary Table 5). *A. myosuroides* showed a higher susceptibility to all powder treatments, whereas *L. multiflorum* displayed higher resilience, particularly towards P_P and P_B, exhibiting FGP values comparable to those of the un-treated control. Notably, P_{EP5} was the only treatment capable of overcoming the resilience of *L. multiflorum*, reducing its FGP to 12%, a level comparable to that observed for *A. myosuroides*.

The development of weed seedlings, in terms of root, shoot and total length (RL, SL and TL, respectively) (**Supplementary Table 6**) was significantly influenced ($p < 0.001$) by both the factors Weed (W) and Powder (P). *L. multiflorum* (W_{Lol}) generally produced longer seedlings compared to *A. myosuroides* (W_{Alo}). Regarding the effect of the powders, P_P was the most inhibitory treatment for both SL and TL, followed by P_{EP5}; while P_B didn't differ from the control (P_0). Interestingly, the RL was affected by all powder treatments, with P_B, P_P and P_{EP5} treated plants showing significant reduction compared to P_0, accounting, on average, for a 23% lower value. In contrast, the biomass partitioning, in terms of root (RDM), shoot (SDM) and total (TDM) dry matter content, showed an inverse trend (**Supplementary Table 6**). A significant effect ($p < 0.05$) of the factor Weed (W) was observed. Despite producing shorter seedlings, *A. myosuroides* showed higher TDM and SDM than *L. multiflorum*, evidencing a more compact tissue structure compared to the more elongated and less dense tissues of the latter. The Powder factor (P) also exhibited a significant effect ($p < 0.001$), with P_{EP5} inducing increase of RDM, SDM and TDM, followed by P_B and P_P. The W x P interaction significantly affected ($p < 0.05$) both SDM and TDM. Specifically, *A. myosuroides* and *L. multiflorum* treated with P_{EP5} (W_{Alo}-P_{EP5} and W_{Lol}-P_{EP5}) reached 30% higher SDM and TDM than their respective controls (W_{Alo}-P_0 and W_{Lol}-P_0). Similarly; *A. myosuroides* treated with P_P (W_{Alo}-P_P) showed 25% and 28% higher SDM and TDM, respectively, than the un-treated control (W_{Alo}-P_0).

The PII and BAI values determined on *A. myosuroides* and *L. multiflorum* are reported in **Supplementary Figure 4**. While the PII didn't vary between the two weeds (**Supplementary Figure 4A**), a significant effect ($p < 0.05$) of the Weed factor (W) was observed on BAI, with *A. myosuroides* showing a higher value (51%) than *L. multiflorum* (31%) (**Supplementary Figure 4B**). The factor Powder (P) significantly influenced ($p < 0.001$) both PII and BAI. Specifically, the highest PII values were observed under the P_P and P_{EP5} treatments, while P_B didn't differ from the un-treated control (P_0) (**Supplementary Figure 4C**). Similarly, the P_P and P_{EP5} treatments significantly increased the BAI value of the weeds compare to P_0 (**Supplementary Figure 4C**).

3.3 *In vivo* allelopathic evaluation: second experiment

3.3.1 Wheat

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Like in the first pot-experiment, also in the second one the factor Weeds (W) didn't affect the germination-related parameters of wheat; whereas the factor powder (P) exerted a significant influence on MGT ($p < 0.001$), SVI ($p < 0.05$), and GI ($p < 0.001$) (**Supplementary Figure 5**). More specifically, P_B and P_P treatments significantly reduced MGT (**Supplementary Figure 5C**) and increased GI for wheat kernel compared to the un-treated control (P_0) (**Supplementary Figure 5C**), indicating a faster and more efficient germination process. Powder treatments also enhanced the SVI, particularly P_P, closely followed by P_{EP5} (**Supplementary Figure 5C**). The W x P interaction only influenced MGT ($p < 0.001$) (**Supplementary Figure 6**). While the MGT value of wheat plants didn't vary among the powder treatments in either the weed-free (W_0) or *L. multiflorum* (W_{Lol}) infested soil, lower values were observed on wheat plants treated with P_B and P_P (averaging 12 days) when grown on soil infested with *A. myosuroides* compared to the un-treated control (P_0) (MGT = 14 days), indicating a more rapid germination.

The growth and dry matter allocation of wheat plants (**Table 4**) were differentially influenced by the experimental factors. Contrary to the first experiment, the effect of Weed (W) significantly influenced the growth-related parameters, particularly SL and TL. More specifically, wheat plants grown in the presence of both *Alopecurus myosuroides* (W_{Alo}) and *Lolium multiflorum* (W_{Lol}) displayed higher SL and TL values than those grown under weed-free conditions (W_0). Conversely, Weed factor (W) didn't exert any significant effect on the root (RDM), shoot (SDM) and total dry matter content (TDM) (**Table 4**). The residual effect of the factor Powder (P) was highly significant ($p < 0.001$) across all growth parameters, with P_{EP5} stimulating the highest SL and TL, similarly to P_P, which also increased RL. Furthermore, P_P emerged as a highly biostimulatory treatment for dry matter accumulation, significantly ($p < 0.001$) increasing RDM and TDM compared to P_0, P_B and P_P. Similarly to P_B, soil treatment with P_P significantly enhanced SDM. The W x P interaction highly affected the three length components (RL, SL and TL) ($p < 0.001$), as well as TDM ($p < 0.05$). Specifically, wheat plants treated with P_P and P_{EP5}, grown on soil infested with *L. multiflorum* (W_{Lol}-P_P and W_{Lol}-P_{EP5}) reached higher TL than the un-treated control (W_{Lol}-P_0), driven by enhanced shoot extension and root development. Regarding dry matter allocation, wheat plants treated with P_P and grown on weed-free soil (W_0-P_P) maximized both TDM compared to weed-free and un-treated soil (W_0-P_0), as well as to plants treated with PEP5, both weed-free and infested (W_0-P_{EP5}, W_{Alo}-P_{EP5} and W_{Lol}-P_{EP5}).

The plant inhibition index (PII) and biomass accumulation inhibition (BAI) displayed distinct trends in the second experiment (**Supplementary Figure 7**). While PII didn't show significant differences across the two factors, W and P, or their interaction (W x P), BAI was significantly influenced by both P ($p < 0.001$) and W x P interaction ($p < 0.05$). Among powders, P_P was the only one to determine a higher BAI value than P_B and P_{EP5} (**Supplementary Figure 7C**), indicating a suppressive effect on biomass accumulation. Regarding the interaction W x P (**Supplementary Figure 7C**), wheat plants grown on weed-free soil treated with P_P (W_0-P_P), as well as those grown on soil infested with *L. multiflorum* and un-treated (W_{Lol}-P_0), exhibited an increase in BAI value, thereby reflecting a marked inhibition of biomass accumulation. In contrast, in soil infested with *L. multiflorum* and treated with P_B and P_{EP5} (W_{Lol}-P_B and W_{Lol}-P_{EP5}), the suppressive trend was inverted, achieving negative BAI values and thus indicating a promotion of biomass accumulation in wheat under competitive conditions.

3.3.2. Weeds

The germination-related parameters detected on weeds during the second *in vivo* experiment are reported in **Table 5**. Only the Weed factor (W) showed a significant influence ($p < 0.001$) across all considered parameters. Specifically, *Lolium multiflorum* (W_{Lol}) exhibited higher FGP, GI and SVI, as well as lower MGT, compared to *Alopecurus myosuroides* (W_{Alo}). Regarding the factor powder (P), a significant effect ($p < 0.05$) on MGT, GI and SVI was observed. All treatments (P_B, P_P and P_{EP5})

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increased SVI relative to the un-treated control (P_0), while P_{EP5} increased the MGT, thereby acting as a germination decelerator, and P_P enhanced the GI reflecting a faster and more synchronized germination process. Finally, the W x P interaction significantly ($p < 0.05$) influenced the SVI, which reached higher values for *L. multiflorum* treated with P_B, P_P and P_{EP5} (W_{Lol}-P_B, W_{Lol}-P_B and W_{Lol}-P_{EP5}, respectively) than for both *A. myosuroides* (W_{Alo}-P_B, W W_{Alo}-P_B and W W_{Alo}-P_{EP5}) and their respective un-treated controls (W_{Alo}-P_0 and W_{Lol}-P_0).

Regarding the growth-related parameters of the two weeds (Supplementary Table 7), a significant effect ($p < 0.001$) of Weed factor (W) on SL and TL was observed, with *L. multiflorum* showing higher SL and TL values than *A. myosuroides*. Also the Powder factor (P) exhibited a significant influence ($p < 0.001$), with P_B showing a promoting residual effect on RL, SL and TL, followed by P_P and P_{EP5}. The W x P interaction didn't show any significant influence on the considered parameters. Considering the dry matter allocation, the powder factor (P) significantly ($p < 0.01$) influenced both SDM and TDM, which increased on soil treated with P_B compared to P_B and P_{EP5}, while not differing from the control (P_0). Finally, the W x P interaction only affected SDM ($p < 0.05$) and TDM ($p < 0.01$) values. *L. multiflorum* grown in untreated soil (W_{Lol}-P_0) reached almost double SDM and TDM compared to *A. myosuroides* grown on un-treated soil (W_{Alo}-P_0), as well as *A. myosuroides* and *L. multiflorum* grown on soil treated with either P_B or P_P (W_{Alo}-P_B, W_{Alo}-P_P, W_{Lol}-P_B and W_{Lol}-P_P), while not differing from *A. myosuroides* and *L. multiflorum* treated with PEP5 (W_{Alo}-P_{EP5}, W_{Lol}-P_{EP5}).

No significant differences were observed between *A. myosuroides* and *L. multiflorum* regarding plant inhibition index (PII) and biomass accumulation inhibition (BAI); on the contrary, both parameters were significantly influenced ($p < 0.01$) by the powder factor (P) (**Supplementary Figure 8**). Specifically, P_B residues in the soil exerted a strong promoting effect on weeds (PII = -31.2%) compared to P_0 and followed by P_P (11.6%) and P_{EP5} (-1.7%) (**Supplementary Figure 8C**). Similarly, a remarkable promoting effect on biomass accumulation was observed in the pots previously treated with P_{EP5}, where weeds reached the lowest BAI value (-4096%) (**Supplementary Figure 8C**). The latter parameters were significantly affected ($p < 0.05$) also by the W x P interaction (**Supplementary Figure 9**), with the most pronounced response recorded for *L. multiflorum* under the P_{EP5} treatment (W_{Lol}-P_{EP5}), which exhibited the lowest BAI value (-6,508%).

4. Discussion

The projected increase in food demand over the coming years has promoted interest in finding sustainable alternatives for food production management. Weeds, together with pathogens and animal pests, reduce crop yield and quality, causing global food security losses ranging between 40-50% (Oerke, 2019). Particularly, weeds represent a pressing management challenge in modern agriculture (Korav et al., 2018). Although the utilization of chemical pesticides has been fundamental in advancing crop protection, exposure to these substances poses a significant risk to human health and the environment. Moreover, their continuous application has accelerated the development of herbicide resistance in weeds (Cullen et al., 2019; Dolatabadi et al., 2021; Montull and Torra, 2023). Thus, a shift towards pest management strategies, based on bio-pesticides with high efficacy and minimal toxicity, is needed (Zhou et al., 2025). Modern agriculture particularly focuses on the application of plant-based products (Rahman et al., 2016). In this context, the present study aimed at evaluating the application of powders from agro-industrial by-products of *Citrus bergamia* and *Punica granatum* for the sustainable management of *Alopecurus myosuroides* and *Lolium multiflorum*, two of the most common weeds affecting durum wheat in the Apulia region. Moreover, the eco-friendly EP5 commercial powder was tested for its herbicidal potential. The study consisted of a preliminary *in vitro* evaluation of plant-derived powder effects on the germination and growth of both wheat kernels and weed seeds, followed by an *in vivo* greenhouse evaluation comprising two consecutive experiments, aimed at evaluating the direct effects of the powders on wheat and weeds, and their residual effects in the soil, respectively.

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The *in vitro* experiment demonstrated a strong allelopathic effect of water extracts from *C. bergamia*, *P. granatum* and EP5 on the germination of the target weeds, whereas wheat was significantly less affected. Among the weeds, *A. myosuroides* proved to be more susceptible than *L. multiflorum*, in terms of mean germination time (MGT) and germination index (GI). Conversely, both weed species exhibited similar sensitivity in terms of final germination percentage (FGP) and seedling vigor index (SVI)**.** These findings align with the study by Abbassi et al. (2025), who reported a high susceptibility of *A. myosuroides* seeds to various plant extracts. Among all treatments, the water extract from the EP5 commercial powder emerged as the most inhibitory agent, achieving near-complete inhibition of all germination-related parameters, followed by *C. bergamia* and *P. granatum*. This potent suppression is likely driven by the chemical composition of EP5, particularly its high citric acid content. In fact, Alpi et al. (2025) confirmed that citric acid highly inhibits weed germination when applied directly onto seeds. In addition to these findings, the present study highlights the minimal inhibitory effects of citric acid on wheat kernels. Furthermore, *C. bergamia* essential oil and *P. granatum* peel organic extracts are well known to suppress weed germination due to their specific phytotoxic profiles (Mohamed and Balah, 2016; Bozhüyük, 2020). The tested extracts also differentially influenced the early growth of wheat and weed seedlings. The root, shoot and total lengths of *L. multiflorum* didn't differ from those of wheat, whereas *A. myosuroides* showed significantly lower values, further confirming its higher sensitivity. On the other hand, the root, shoot and total dry matter of both weed species were statistically lower than those of wheat, underscoring the higher tolerance of the crop over the two weeds. While the overall dry matter accumulation was comparable between *A. myosuroides* and *L. multiflorum*, their root systems responded differently; specifically, *A. myosuroides* roots were more sensitive to biomass reduction than those of *L. multiflorum*. Although *C. bergamia*, *P. granatum* and EP5 caused similar reductions in seedling length, EP5 exerted the strongest suppressive effect on shoot elongation. Regarding biomass, EP5 proved to be the only treatment that significantly inhibited overall plant growth. The biomass reduction induced by *C. bergamia* on root, shoot, and total dry matter was slightly more pronounced than *P. granatum*; notably, *P. granatum* did not alter root dry matter. These growth responses are consistent with previous findings. Alpi et al. (2025) verified that citric acid reduces weed development, while Caputo et al. (2020) demonstrated that crop seedlings tolerate *C. bergamia* essential oil better than weeds. Furthermore, *P. granatum* was the only treatment that reduced seedling length without affecting dry matter, in accordance with the observations of Emam (2022) and Rasul (2024), who reported that *P. granatum* aqueous extracts significantly inhibit plant elongation but exert no phytotoxic effects on total biomass weight. These length and biomass trends perfectly match the observed data on plant inhibition index and biomass accumulation inhibition. While wheat and *L. multiflorum* shared a similar tolerance to the powders, *A. myosuroides* proved to be the most vulnerable species. However, regarding biomass accumulation, both the crop and the weeds were highly susceptible across all extract treatments. EP5 proved to be more phytotoxic than the other extracts in terms of plant inhibition index. Beyond germination- and growth-related parameters, the extracts also altered root architecture, specifically affecting root number per seedling and individual root length. Overall, wheat kernels generated a higher number of roots than weed seeds. The exposure to the extracts significantly shortened individual root length across the weed species, whereas wheat consistently maintained the highest values, confirming its lower susceptibility to the tested extracts. Moreover, while root elongation was reduced by all water extracts, the number of roots per seedling was affected only by *P. granatum* and EP5, with *C. bergamia* showing no allelopathic effect.

The findings of the first *in vivo* experiment confirmed the allelopathic inhibitory potential of the tested powders on the two target weeds. Specifically, also in this trial, *L. multiflorum* seeds performed better than *A. myosuroides* across all germination-related parameters, except when treated with EP5, suggesting that this product effectively nullified the competitive differences between the two weed species. This species-specific variation in weed susceptibility to plant-based powders aligns with recent findings where different grass weeds showed distinct sensitivity thresholds to plant-derived

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bioherbicides (Leoni et al., 2025). Interestingly, none of the germination-related parameters were altered when wheat kernels were sown in the presence of the weeds. This lack of interaction indicates that the weeds were not competitive against wheat at this stage, as the powder treatments inhibited weed germination far more severely than crop germination. Similarly, the powder treatments, particularly *C. bergamia* and *P. granatum*, did not affect the germination or seed vigor of wheat, although they increased the mean germination time and decreased the germination index. The safety of *C. bergamia* and *P. granatum* subproducts on grain crops, alongside their simultaneous phytotoxicity against target weeds, has been documented by other authors who observed weed suppression without compromising main crop establishment (Caputo et al., 2020; Rasul et al., 2024). Notably, EP5 was the only treatment that adversely affected all germination-related parameters in wheat as well. This pronounced, non-selective suppression is consistent with recent pre-emergence trials involving organic acids, which demonstrate that high concentrations of citric acid can significantly delay or suppress shoot emergence even in tolerant crops like soft wheat (Alpi et al., 2025). Expectedly, the root and shoot lengths of both wheat and weeds were greater in the *in vivo* trial than in the *in vitro* experiment, as the latter was conducted for 14 days within a highly restricted growing space, whereas the *in vivo* study was carried out in pots and extended over 45 days. Particularly, *L. multiflorum* proved to be more competitive than *A. myosuroides* against wheat, consistently exhibiting higher root, shoot, and total lengths. Regarding the root elongation, wheat was mostly affected by EP5, followed to a lesser extent by *C. bergamia*, whereas *P. granatum* showed almost no allelopathic effects. The marked sensitivity of wheat root elongation to EP5 is likely due to its citric acid content aligning with recent greenhouse observations where pre-emergence applications of organic acid, including citric acid, significantly reduced early root expansion of four weed species (Alpi et al., 2025). In contrast, weed roots were considerably more sensitive to the treatments, with all tested powders causing significant length reductions. Interestingly, *C. bergamia* promoted wheat shoot elongation compared to the untreated control, whereas the other two treatments reduced shoot growth, with EP5 being the most suppressive. This selective response highlights a species-specific sensitivity to *Citrus* secondary metabolites, which can exert differential allelopathic effects-ranging from growth inhibition in weeds to slight biostimulation in crops-depending on the botanical target (Caputo et al., 2020). The weeds followed a different trend: the most severe restriction on weed shoot length was determined by *P. granatum*, followed by EP5, whereas *C. bergamia* didn't affect weed shoot elongation. The strong, suppressive effect of pomegranate is consistent with studies reporting that aqueous extracts of *P. granatum* peel significantly impair both radicle and plumule development in invasive grass species (Rasul, 2024). For both the crops and the weeds, total plant length was predominantly driven by shoot development. Consequently, the most inhibitory treatments for total wheat length were EP5 followed by *P. granatum*, reflecting the trends observed in the shoots. For the weeds, however, a reverse order was detected, with *P. granatum* being the most inhibitory agent, followed by EP5. *C. bergamia* was the only treatment that exerted no significant influence, positive or negative, on the total length of the plants. The *in vivo* biomass accumulation for wheat didn't vary in the presence of the two weeds. Furthermore, contrary to the length trends, *A. myosuroides* accumulated more biomass than *L. multiflorum*, particularly regarding shoot and total dry matter. Wheat and weeds also displayed contrasting dry matter responses to the treatments. For wheat, *P. granatum* was the only treatment that reduced dry matter accumulation across the roots, shoots, and the whole plant, with EP5 and *C. bergamia* exerting no significant effect compared to the untreated control. Conversely, the EP5 treatment increased the root, shoot, and total dry matter of the weeds. Overall, EP5 exhibited a promoting effect on weed dry matter accumulation, likely due to the biostimulant or growth-promoting action of the citric acid contained in the powder. This is in accordance with Ibrahim (2023), who reported that the application of citric acid to the soil significantly increases dry weight and overall development of different plants. On the other hand, the biomass reduction induced by the other treatments matches Othman et al. (2023), who observed that *P. granatum* aqueous extracts significantly reduce the *in vivo* weight of young seedlings. Regarding seedling vigor and biomass accumulation, the overall inhibition levels of these two parameters decreased under *in*

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vivo conditions. This reduction in phytotoxicity is widely attributed to the buffering capacity of the soil matrix, which adsorbs and partially degrades allelochemicals over time, thereby mitigating the severe suppression typically observed in preliminary laboratory assays (Scavo et al., 2019). Once again, the presence of weeds didn't alter the vigor or biomass accumulation of wheat plants, confirming the higher physiological resilience and selective tolerance of grain crops against plant-derived herbicides during early growth stages (Maurya et al., 2024). Furthermore, while both weed species shared an identical vigor response to the treatments, *L. multiflorum* biomass accumulation was less inhibited than that of *A. myosuroides*. The treatments exerted highly differentiated effects on wheat and weed development. Overall, the powder treatments inhibited seedling vigor; wheat was most suppressed by EP5, less by *P. granatum*, and slightly promoted by *C. bergamia*, whereas the weeds were inhibited by all three treatments, with *C. bergamia* being the least inhibitory. Regarding biomass accumulation, the trends were completely different. In fact, wheat biomass accumulation was promoted, though not significantly, by EP5 and *C. bergamia*, but inhibited by *P. granatum*. Conversely, weed biomass accumulation was suppressed by all treatments, with EP5 being the most inhibitory agent, followed by *P. granatum* and *C. bergamia*. Ultimately, wheat proved to be less sensitive to the treatments than the two weeds in terms of both vigor and biomass accumulation.

The findings of the second *in vivo* experiment regarding germination parameters revealed no allelopathic residual effects on wheat, with such effects being almost completely absent for the weeds as well. Consistent with the first trial, weed competition didn't affect any of the germination parameters of wheat plants. Moreover, *L. multiflorum* performance remained higher than *A. myosuroides*. Interestingly, the powders reduced the mean germination time of both wheat and weeds, while simultaneously increasing their germination index. Furthermore, the powder treatments enhanced the seedling vigor index of wheat, with *P. granatum* exerting the highest promoting effect, as well as that of the weeds, which were consistently stimulated by all powder residues. This response indicates a loss of the initial allelopathic inhibition over time. The degradation of the bioactive compounds in the soil matrix likely reduced their concentration below the toxicity threshold, turning the potential bioherbicides into growth biostimulants that enhanced both the speed and overall success of germination. This phenomenon is consistent with the process of soil-mediated allelochemical dissipation, where the progressive breakdown of phytotoxic molecules reduces their bioavailability below threshold levels, often triggering a hormetic response that biostimulates early plant development (Scavo et al., 2019). The residual effects of the powders promoted the shoot length of both wheat and weeds, marking a general increase in elongation compared to the first experiment, with the sole exception of *C. bergamia* on wheat. This widespread improvement suggests that as the primary phytotoxic molecules degraded, the remaining organic nutrients from the residues began to act as growth biostimulants (Consoli et al., 2023). Regarding dry matter accumulation, wheat plants showed no significant differences whether grown with or without weeds, confirming that the weed species were not competitive enough to suppress the crop. Furthermore, no significant differences were observed between the two weeds. Among the treatments, *P. granatum* promoted wheat dry matter, enhancing both shoot and root dry matter content. Similarly, *P. granatum* increased the total dry matter of the weeds, particularly enhancing root accumulation. This response suggests that the pomegranate residues lost their initial allelopathic toxicity over time, transitioning into a nutritional or biostimulant source that favored overall biomass accumulation (Rasul, 2024). In general, *P. granatum* residues promoted both wheat length and dry matter, while *C. bergamia* enhanced weed length, and *P. granatum* stimulated weed dry matter accumulation. Conversely, EP5 was the only treatment that maintained a slight inhibition on plant growth over time. Regarding the final indices, both the plant vigor inhibition and biomass accumulation index were consistently negative for both wheat and weeds, and even more negative compared to the first experiment, underscoring that the residual effects of the treatments induced a higher vegetative stimulation rather than suppression.

5. Conclusions

The powders from *Citrus bergamia* and *Punica granatum* peels used in the present study, along with the sustainable commercial product EP5, have already demonstrated remarkable potential in managing fungal pathogens in wheat and tomato under both *in vitro* and *in vivo* conditions (Conte et al., 2026a; Conte et al., 2026b). The present findings confirm the potential of these matrices also for the management of *Alopecurus myosuroides* and *Lolium multiflorum*, the two main weeds affecting wheat crops. This suggests their potential for effective weed management, also in line with the current agricultural policies that strongly promote the adoption of eco-friendly products and a significant reduction in chemical inputs. Beyond providing an herbicidal activity, the valorization of crop by-product powders aligns with the principles of the circular economy, transforming agricultural waste into high-value bio-based formulations for modern weed management.

Since these natural products also exerted a slight allelopathic effect on wheat kernels, although considerably lower than that observed on the target weeds, they are particularly suitable for pre-emergence weed treatments. Applying these products prior to weed emergence ensures high efficacy against both seed germination and early seedling development. Moreover, because these powders lose their phytotoxic potential against weeds over time due to soil degradation, performing the treatment well ahead of wheat sowing ensures that any residual phytotoxic effects do not negatively impact crop establishment and growth.

Interestingly, the tested treatments displayed highly differentiated effects on wheat and weed species, with certain soil-mediated residual effects even promoting wheat vegetative parameters over time. Given that the in vitro assays indicated the allelopathic inhibitory potential of water extracts from raw powders, an alternative management strategy could involve post-emergence foliar spray applications on young weed seedlings. Consequently, the next steps of this research will focus on evaluating the efficacy of these different application methods under open-field conditions. Additionally, a detailed characterization of the chemical profiles of these powders is required to identify the specific bioactive compounds responsible for the observed weed inhibition.

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6. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions

AL: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft preparation, Writing - review & editing, Visualization, Supervision. TC: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft preparation, Writing - review &editing, Visualization. MGM: Methodology, Software, Validation, Investigation, Data curation, Visualization. AC: Software, Validation, Investigation, Writing - original draft preparation, Writing - review & editing, Visualization, Supervision.

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Figures and Tables

Figure 1. Root length (RL), shoot length (SL) and total length (TL) of emerged seedlings of wheat and weeds in the *in vitro* experiment, as affected by the factors, Species (S) **(A, B and C)** and Extract (E) **(D, E and F)**. W, wheat; Alo, *A. myosuroides*; Lol, *L. multiflorum*; E_0, distilled water or control; E_B, water extracts from bergamot peel powder, E_P, water extracts from pomegranate peel powder; E_{EP5}, water extracts from EP5 commercial powder. Values are means (n=4) ± standard error. Different letters above bars indicate significant differences among species and extracts ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

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Table 1. Effect of the factors Species (S), Extract (E) and the interaction Species x Extract (S × E) on the final germination percentage (FGP), mean germination time (MGT), germination index (GI) and seed vigor index (SVI) of wheat (W) kernels, *A. myosuroides* (Alo) and *L. multiflorum* (Lol) seeds, treated with distilled water (E_0 or control) and water extracts from bergamot peel powder (E_B), pomegranate peel powder (E_P) and EP5 commercial powder (E_{EP5}), in the *in vitro* experiment.

Experimental factor	FGP (%)	MGT (days)	GI	SVI
Species (S)				
W	38.0 ± 10.1 a	5.2 ± 1.1 c	7.2 ± 2.6 a	471.9 ± 179.6 a
Alo	16.2 ± 7.4 b	11.3 ± 0.8 a	0.7 ± 0.3 c	136.8 ± 64.4 b
Lol	21.0 ± 7.0 b	8.5 ± 1.1 b	1.8 ± 0.7 b	239.9 ± 99.1 b
Extract (E)				
E_0	78.0 ± 5.1 a	3.5 ± 0.6 c	11.3 ± 2.8 a	1013.4 ± 156.4 a
E_B	7.7 ± 2.3 bc	8.6 ± 1.2 b	0.5 ± 0.2 bc	50.9 ± 20.5 b
E_P	13.7 ± 5.8 b	8.7 ± 1.3 b	1.2 ± 0.7 b	66.5 ± 37.0 b
E_{EP5}	1.0 ± 1.0 c	12.6 ± 0.4 a	0.0 ± 0.0 c	0.6 ± 0.6 b
Species x Extract (S x E)				
W-E_0	100.0 ± 0.00 a	1.1 ± 0.0 ef	24.2 ± 0.3 a	1601.5 ± 248.3 a
Alo- E_0	65.0 ± 5.2 b	6.1 ± 0.4 b-d	2.8 ± 0.3 c	547.2 ± 89.5 bc
Lol- E_0	69.0 ± 4.1 b	3.2 ± 0.4 d-f	6.7 ± 0.2 b	891.6 ± 81.8 b
W-E_B	16.0 ± 2.8 cd	4.7 ± 0.3 c-e	1.1 ± 0.2 cd	118.5 ± 43.4 d
Alo- E_B	0.0 ± 0.0 d	nd	0.0 ± 0.0 d	0.0 ± 0.0 d
Lol- E_B	7.0 ± 3.0 d	8.0 ± 1.9 -a-c	0.3 ± 0.16 d	34.3 ± 16.3 d
W-E_P	33.0 ± 12.3 c	3.5 ± 0.6 d-f	3.4 ± 1.6 c	165.8 ± 97.7 cd
Alo- E_P	0.0 ± 0.0 d	nd	0.0 ± 0.0 d	0.0 ± 0.0 d
Lol- E_P	8.0 ± 4.6 d	9.6 ± 1.9 ab	0.3 ± 0.2 d	33.8 ± 19.6 d
W-E_{EP5}	3.0 ± 3.0 d	11.8 ± 1.2 a	0.1 ± 0.1 d	1.8 ± 1.8 d
Alo- E_{EP5}	0.0 ± 0.0 d	nd	0.0 ± 0.0 d	0.0 ± 0.0 d
Lol- E_{EP5}	0.0 ± 0.0 d	nd	0.00 ± 0.0 d	0.0 ± 0.0 d
Significance				
S	***	***	***	***
E	***	***	***	***
S x E	***	***	***	***

Values are means (n=4) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$. nd, not detected.

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928 **Table 2.** Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on
929 the final germination percentage (FGP), mean germination time (MGT), germination index (GI) and
930 seed vigor index (SVI) of wheat, grown on soil weed-free (W_0 or control) and infested with *A.*
931 *myosuroides* (W_{Alo}) and *L. multiflorum* (W_{Lol}), un-treated (P_0 or control) and treated with bergamot
932 peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the first *in*
933 *vivo* experiment.

Experimental factor	FGP (%)	MGT (days)	GI	SVI
Weed (W)				
W_0	77.5 ± 2.9 a	14.9 ± 0.3 a	1.8 ± 0.1 a	2301.6 ± 108.3 a
W_{Alo}	79.6 ± 2.6 a	14.8 ± 0.3 a	1.8 ± 0.1 a	2429.5 ± 94.1 a
W_{Lol}	81.5 ± 2.4 a	15.0 ± 0.3 a	1.8 ± 0.1 a	2287.1 ± 77.9 a
Powder (P)				
P_0	90.1 ± 1.1 a	11.9 ± 0.2 c	2.5 ± 0.0 a	2804.0 ± 58.7 a
P_B	89.9 ± 0.9 a	15.0 ± 0.2 b	2.0 ± 0.0 b	2656.0 ± 52.2 a
P_P	88.2 ± 1.0 a	15.8 ± 0.2 b	1.8 ± 0. b	2604.3 ± 43.0 a
P_{EP5}	49.9 ± 3.1 b	16.9 ± 0.4 a	1.0 ± 0.1 c	1293.4 ± 92.2 b
Weed x Powder (W x P)				
W_0-P_0	87.9 ± 1.8 a	11.9 ± 0.3 d	2.5 ± 0.0 a	2862.3 ± 119.9 a
W_{Alo}-P_0	90.6 ± 1.7 a	12.0 ± 0.3 d	2.5 ± 0.0 a	2885.4 ± 93.8 a
W_{Lol}-P_0	91.7 ± 2.1 a	12.0 ± 0.3 d	2.5 ± 0.1 a	2664.3 ± 84.9 a
W_0-P_B	90.8 ± 1.9 a	14.5 ± 0.1 c	2.0 ± 0.0 a	2616.7 ± 107.0 a
W_{Alo}-P_B	88.7 ± 1.5 a	14.9 ± 0.3 bc	1.9 ± 0.0 a	2841.8 ± 74.5 a
W_{Lol}-P_B	90.2 ± 1.3 a	15.5 ± 0.3 bc	1.9 ± 0.0 a	2509.4 ± 68.7 a
W_0-P_P	88.3 ± 1.8 a	15.2 ± 0.2 bc	1.9 ± 0.0 a	2704.8 ± 55.3 a
W_{Alo}-P_P	87.1 ± 2.1 a	16.4 ± 0.5 abc	1.7 ± 0.0 a	2562.1 ± 102.8 a
W_{Lol}-P_P	89.2 ± 1.2 a	15.7 ± 0.5 bc	1.9 ± 0.0 a	2545.9 ± 52.7 a
W_0-P_{EP5}	42.9 ± 5.2 a	18.1 ± 0.8 a	0.8 ± 0.1 a	1022.6 ± 144.3 b
W_{Alo}-P_{EP5}	51.9 ± 6.1 a	16.0 ± 0.7 abc	1.1 ± 0.1 a	1428.9 ± 175.3 b
W_{Lol}-P_{EP5}	54.8 ± 4.8 a	16.7 ± 0.6 ab	1.1± 0.1 a	1428.9 ± 144.8 b
Significance				
W	ns	ns	ns	ns
P	***	***	***	***
W x P	ns	*	ns	*

934 Values are means (n=16) ± standard error. In each column, means followed by the same letters are
935 not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

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Table 3. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W × P) on the root (RL), shoot (SL) and total length (TL), root (RDM), shoot (SDM) and total dry matter content (TDM) of wheat, grown on soil weed-free (W_0 or control) and infested with *A. myosuroides* (W_{Alo}) and *L. multiflorum* (W_{Lol}), un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the first *in vivo* experiment.

Experimental factor	RL	SL	TL	RDM	SDM	TDM
	(cm)			(% fw)		
Weed (W)						
W_0	11.3 ± 0.1 a	19.8 ± 0.1 a	31.2 ± 0.2 a	24.1 ± 1.5 a	22.5 ± 1.0 a	22.4 ± 1.0 a
W_{Alo}	10.9 ± 0.1 a	19.6 ± 0.1 a	30.5 ± 0.2 a	28.1 ± 1.9 a	20.9 ± 0.6 a	22.0 ± 0.6 a
W_{Lol}	10.3 ± 0.1 b	18.8 ± 0.1 b	29.1 ± 0.2 b	26.5 ± 1.2 a	21.0 ± 0.5 a	22.2 ± 0.5 a
Powder (P)						
P_0	11.5 ± 0.1 a	20.0 ± 0.1 b	31.5 ± 0.2 a	26.4 ± 1.5 ab	23.0 ± 0.8 ab	23.2 ± 0.7 ab
P_B	10.8 ± 0.1 b	21.3 ± 0.1 a	32.1 ± 0.2 a	26.9 ± 1.8 ab	20.7 ± 0.7 bc	21.8 ± 0.7 b
P_P	11.1 ± 0.1 ab	18.4 ± 0.1 c	29.5 ± 0.2 b	20.7 ± 0.9 b	18.5 ± 0.2 c	19.0 ± 0.3 c
P_{EP5}	9.1 ± 0.2 c	16.8 ± 0.2 d	25.9 ± 0.3 c	30.9 ± 2.3 a	23.8 ± 1.2 a	24.7 ± 1.2 a
Weed x Powder (W x P)						
W_0-P_0	12.0 ± 0.2 a	20.4 ± 0.1 b	32.4 ± 0.3 b	27.2 ± 3.1 a	23.8 ± 1.5 ab	24.1 ± 1.5 ab
W_{Alo}-P_0	11.4 ± 0.2 a-c	20.4 ± 0.1 b	31.8 ± 0.3 bc	28.1 ± 2.3 a	23.2 ± 1.7 b	23.7 ± 1.1 a-c
W_{Lol}-P_0	11.3 ± 0.2 a-d	19.1 ± 0.1 c	30.3 ± 0.3 d	23.9 ± 2.3 a	21.9 ± 0.8 b	21.9 ± 0.9 bc
W_0-P_B	11.9 ± 0.2 a	22.2 ± 0.2 a	34.2 ± 0.3 a	20.8 ± 1.4 a	18.2 ± 0.7 b	19.0 ± 0.8 bc
W_{Alo}-P_B	10.3 ± 0.2 c-e	21.7 ± 0.2 a	32.0 ± 0.3 b	29.2 ± 4.2 a	20.5 ± 1.1 b	21.6 ± 1.3 bc
W_{Lol}-P_B	10.3 ± 0.2 de	20.0 ± 0.2 b	30.2 ± 0.3 d	30.6 ± 2.6 a	23.2 ± 1.6 b	24.8 ± 1.4 ab
W_0-P_P	11.7 ± 0.2 a	18.9 ± 0.2 c	30.6 ± 0.3 cd	17.0 ± 0.4 a	18.3 ± 0.3 b	17.7 ± 0.3 c
W_{Alo}-P_P	11.5 ± 0.3 ab	18.0 ± 0.2 de	29.4 ± 0.4 de	21.0 ± 1.5 a	18.1 ± 0.3 b	19.0 ± 0.5 bc
W_{Lol}-P_P	10.2 ± 0.2 e	18.3 ± 0.2 cd	28.5 ± 0.3 ef	24.1 ± 1.8 a	19.1 ± 0.5 b	20.4 ± 0.8 bc
W_0-P_{EP5}	8.2 ± 0.3 f	15.6 ± 0.3 f	23.8 ± 0.5 h	31.3 ± 4.2 a	29.5 ± 2.7 a	28.9 ± 2.8 a
W_{Alo}-P_{EP5}	10.3 ± 0.3 b-e	17.1 ± 0.2 e	27.4 ± 0.4 fg	34.0 ± 5.2 a	21.8 ± 1.2 b	23.6 ± 1.5 a-c
W_{Lol}-P_{EP5}	8.8 ± 0.3 f	17.4 ± 0.3 de	26.1 ± 0.5 g	27.5 ± 2.4 a	20.0 ± 0.8 b	21.6 ± 0.8 bc
Significance						
W	***	***	***	ns	ns	ns
P	***	***	***	***	***	***
W x P	***	***	***	ns	***	***

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

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Table 4. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on the root (RL), shoot (SL) and total length (TL), root (RDM), shoot (SDM) and total dry matter content (TDM) of wheat, grown on weed-free soil (W_0 or control) and infested with *A. myosuroides* (WAlo) and *L. multiflorum* (WLol) soil, un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the second *in vivo* experiment.

Experimental factor	RL	SL	TL	RDM	SDM	TDM
	(cm)			(% fw)		
Weed (W)						
W_0	8.6 ± 0.1 b	19.1 ± 0.1 b	27.7 ± 0.2 b	35.2 ± 2.1 a	34.8 ± 1.5 a	34.4 ± 1.6 a
W_{Alo}	8.9 ± 0.1 ab	19.7 ± 0.2 a	28.6 ± 0.2 a	32.2 ± 2.1 a	36.5 ± 1.9 a	34.5 ± 1.7 a
W_{Lol}	9.0 ± 0.1 a	20.1 ± 0.2 a	29.1 ± 0.2 a	37.3 ± 3.6 a	38.0 ± 2.2 a	36.7 ± 2.4 a
Powder (P)						
P_0	9.7 ± 0.1 a	18.1 ± 0.2 c	27.8 ± 0.2 b	32.5 ± 2.9 b	33.7 ± 1.8 ab	32.8 ± 1.9 b
P_B	8.3 ± 0.1 b	19.3 ± 0.2 b	27.6 ± 0.2 b	30.2 ± 2.0 b	37.9 ± 2.0 a	34.1 ± 1.6 b
P_P	9.0 ± 0.1 a	20.1 ± 0.2 a	29.0 ± 0.2 a	47.4 ± 3.9 a	42.3 ± 2.5 a	43.2 ± 2.8 a
P_{EP5}	8.1 ± 0.1 b	20.5 ± 0.2 a	28.6 ± 0.2 a	29.8 ± 1.1 b	30.2 ± 1.5 b	29.9 ± 1.3 b
Weed x Powder (W x P)						
W_0-P_0	10.2 ± 0.2 a	17.4 ± 0.2 d	27.6 ± 0.3 cd	26.3 ± 2.4 a	27.4 ± 1.6 a	26.8 ± 1.4 c
W_{Alo}-P_0	9.7 ± 0.3 a-c	18.9 ± 0.4 c	28.6 ± 0.5 b-d	29.8 ± 5.6 a	37.5 ± 2.0 a	34.1 ± 2.9 a-c
W_{Lol}-P_0	8.6 ± 0.3 c-g	18.7 ± 0.4 cd	27.2 ± 0.5 cd	47.4 ± 7.3 a	42.5 ± 4.4 a	43.5 ± 5.0 ab
W_0-P_B	7.6 ± 0.1 gh	19.3 ± 0.2 c	26.9 ± 0.3 d	31.6 ± 2.7 a	36.1 ± 2.8 a	34.0 ± 2.4 a-c
W_{Alo}-P_B	9.4 ± 0.3 a-d	19.5 ± 0.3 bc	28.9 ± 0.5 abc	29.3 ± 2.6 a	40.2 ± 4.2 a	35.2 ± 2.6 a-c
W_{Lol}-P_B	8.5 ± 0.2 d-g	19.0 ± 0.3 c	27.6 ± 0.4 cd	28.3 ± 5.4 a	39.2 ± 3.9 a	33.1 ± 3.4 a-c
W_0-P_P	8.4 ± 0.2 e-g	19.8 ± 0.3 bc	28.2 ± 0.3 cd	52.1 ± 5.8 a	44.4 ± 3.4 a	46.0 ± 3.9 a
W_{Alo}-P_P	9.3 ± 0.3 a-e	19.6 ± 0.3 bc	28.9 ± 0.5 abc	38.4 ± 5.2 a	38.9 ± 5.3 a	38.6 ± 5.1 a-c
W_{Lol}-P_P	9.8 ± 0.3 ab	21.2 ± 0.3 ab	31.0 ± 0.4 a	46.7 ± 9.2 a	41.4 ± 5.9 a	42.2 ± 6.5 a-c
W_0-P_{EP5}	8.2 ± 0.2 f-h	19.9 ± 0.3 bc	28.0 ± 0.4 cd	30.5 ± 1.6 a	31.3 ± 2.6 a	30.8 ± 2.1 bc
W_{Alo}-P_{EP5}	7.2 ± 0.2 h	20.8 ± 0.4 ab	28.0 ± 0.5 cd	31.4 ± 2.7 a	29.4 ± 2.6 a	30.0 ± 2.6 bc
W_{Lol}-P_{EP5}	8.9 ± 0.2 b-f	21.3 ± 0.4 a	30.2 ± 0.5 ab	26.8 ± 1.9 a	28.9 ± 1.4 a	28.1 ± 1.5 bc
Significance						
W	ns	***	***	ns	ns	ns
P	***	***	***	***	***	***
W x P	***	***	***	ns	ns	*

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

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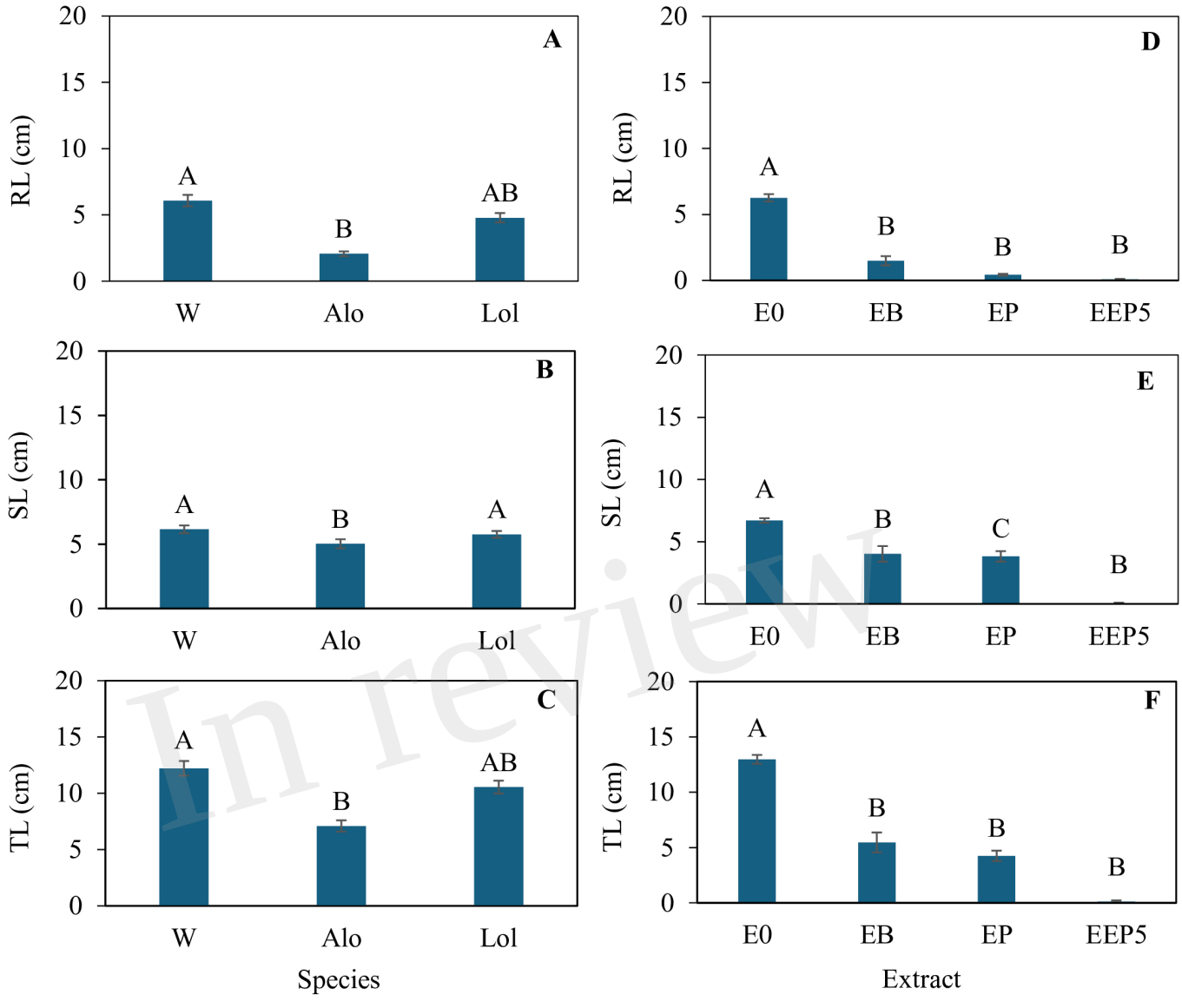
Table 5. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on the final germination percentage (FGP), mean germination time (MGT), germination index (GI) and seed vigor index (SVI) of the two weeds, *A. myosuroides* (Alo) and *L. multiflorum* (Lol), grown on soil un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the second *in vivo* experiment.

Experimental factor	FGP (%)	MGT (days)	GSI	SVI
Weed (W)				
Alo	23.0 ± 2.4 b	17.7 ± 0.8 a	0.5 ± 0.1 b	461.4 ± 47.2 b
Lol	53.5 ± 2.0 a	13.4 ± 0.2 b	1.4 ± 0.0 a	1336.1 ± 55.6 a
Powder (P)				
P_0	32.9 ± 4.8 a	14.4 ± 0.4 b	0.8 ± 0.1 b	684.3 ± 103.3 b
P_B	36.9 ± 5.2 a	15.0 ± 0.7 b	1.0 ± 0.1 ab	973.2 ± 145.4 a
P_P	40.4 ± 5.7 a	14.6 ± 0.8 b	1.1 ± 0.1 a	944.2 ± 159.0 a
P_{EP5}	42.9 ± 3.9 a	18.2 ± 1.5 a	0.9 ± 0.1 ab	993.1 ± 108.5 a
Weed x Powder (W x P)				
W_{Alo}-P_0	17.9 ± 3.8 a	15.5 ± 0.6 a	0.4 ± 0.1 a	337.7 ± 81.5 d
W_{Lol}-P_0	47.9 ± 4.3 a	13.3 ± 0.2 a	1.2 ± 0.1 a	1031.0 ± 69.0 bc
W_{Alo}-P_B	20.8 ± 5.1 a	17.0 ± 0.9 a	0.4 ± 0.1 a	481.9 ± 114.5 d
W_{Lol}-P_B	52.9 ± 4.3 a	13.0 ± 0.3 a	1.5 ± 0.1 a	1464.6 ± 92.0 a
W_{Alo}-P_P	19.6 ± 2.1 a	16.7 ± 1.0 a	0.8 ± 0.1 a	381.9 ± 52.0 d
W_{Lol}-P_P	61.2 ± 3.2 a	12.4 ± 0.3 a	1.5 ± 0.1 a	1506.6 ± 123.6 a
W_{Alo}-P_{EP5}	33.8 ± 5.7 a	21.4 ± 2.4 a	0.4 ± 0.1 a	644.1 ± 95.4 cd
W_{Lol}-P_{EP5}	52.1 ± 3.1 a	14.9 ± 0.6 a	1.4 ± 0.1 a	1342.1 ± 80.7 ab
Significance				
W	***	***	***	***
P	ns	*	*	*
W x P	ns	ns	ns	*

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

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Figure 1.JPEG



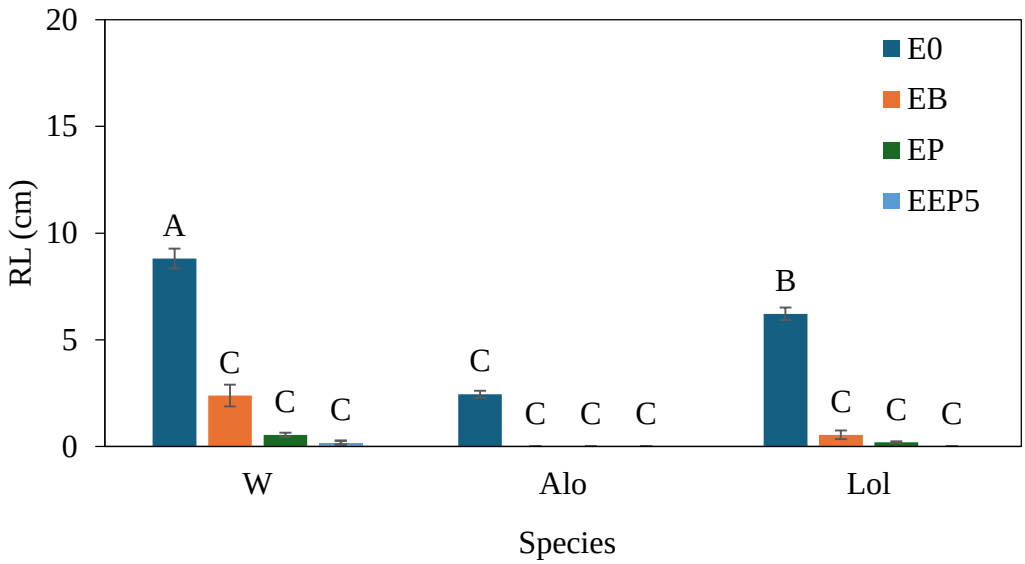
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Supplementary Table 1. Treatment combinations used in the *in vitro* experiment.

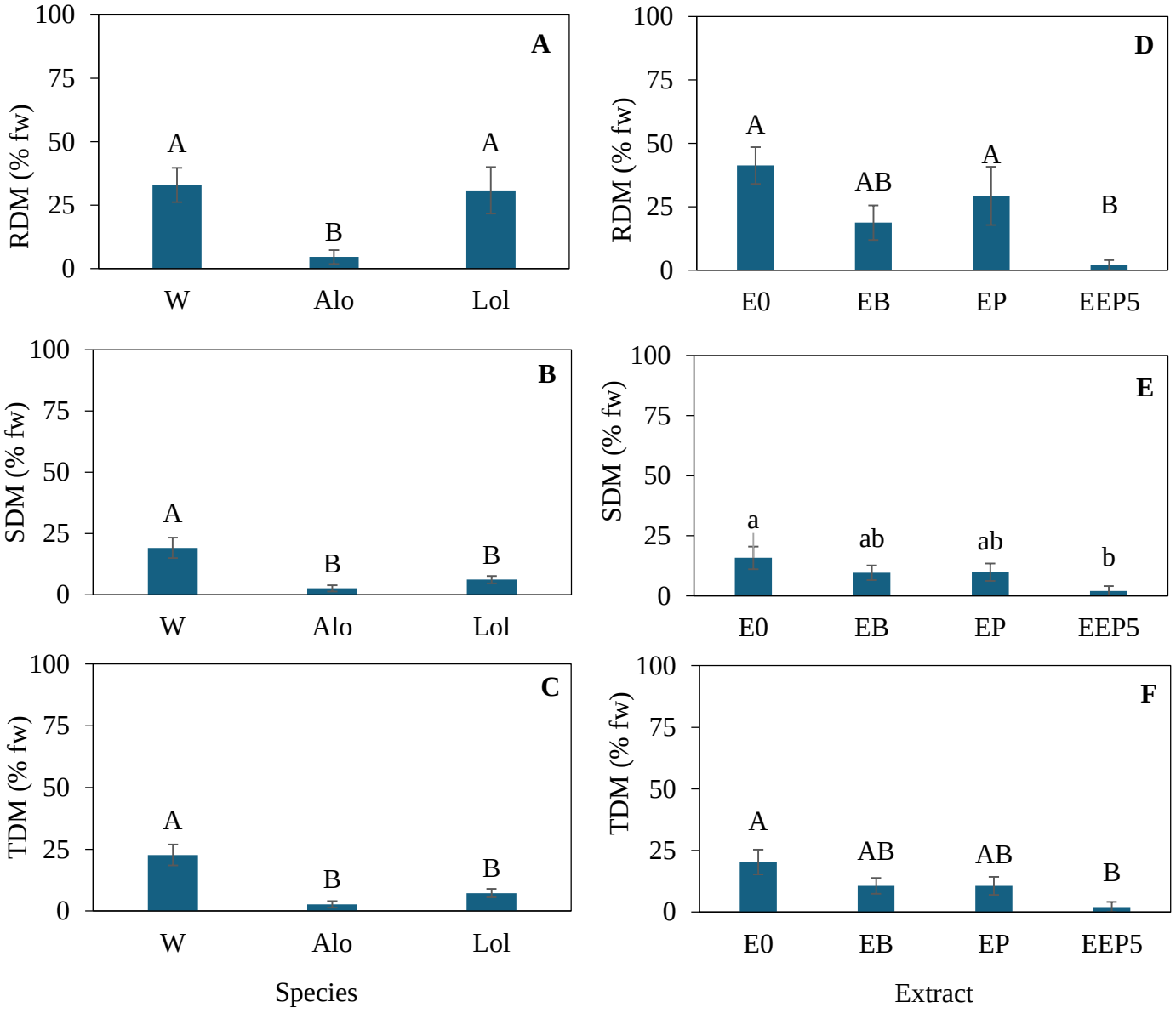
Species (S)	Extract (E)	Abbreviation
Wheat	Distilled water	W-E_0 (control)
A. myosuroides	Distilled water	Alo-E_0
L. multiflorum	Distilled water	Lol-E_0
P. rhoeas	Distilled water	Pap-E_0
Wheat	Bergamot	W-E_B
A. myosuroides	Bergamot	Alo -E_B
L. multiflorum	Bergamot	Lol -E_B
P. rhoeas	Bergamot	Pap-E_B
Wheat	Pomegranate	W-E_P
A. myosuroides	Pomegranate	Alo-E_P
P. rhoeas	Pomegranate	Pap-E_P
L. multiflorum	Pomegranate	Lol-E_P
Wheat	EP5	W-E_{EP5}
A. myosuroides	EP5	Alo-E_{EP5}
L. multiflorum	EP5	Lol-E_{EP5}
P. rhoeas	EP5	Pap-E_{EP5}

Supplementary Table 2. Treatment combinations used in the first and second *in vivo* experiments.

Weed (W)	Powder (P)	Abbreviation
-	-	W_0-P_0 (control)
A. myosuroides	-	W_{Alo}-P_0
L. multiflorum	-	W_{Lol}-P_0
-	Bergamot	W_0-P_B
A. myosuroides	Bergamot	W_{Alo}-P_B
L. multiflorum	Bergamot	W_{Lol}-P_B
-	Pomegranate	W_0-P_P
A. myosuroides	Pomegranate	W_{Alo}-P_P
L. multiflorum	Pomegranate	W_{Lol}-P_P
-	EP5	W_0-P_{EP5}
A. myosuroides	EP5	W_{Alo}-P_{EP5}
L. multiflorum	EP5	W_{Lol}-P_{EP5}



Supplementary Figure 1. Root length (RL) of emerged seedlings of wheat and weeds in the *in vitro* experiment, as affected by the interaction Species x Extract (S x E). W: wheat; Alo: *A. myosuroides*; Lol: *L. multiflorum*; E_0: distilled water or control; E_B: water extracts from bergamot peel powder, E_P: water extracts from pomegranate peel powder; E_{EP5}: water extracts from EP5 commercial powder. Values are means (n=4) ± standard error. Different letters above bars indicate significant differences among species and extracts ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.



Supplementary Table 2. Root dry matter (RDM), shoot dry matter (SDM) and total dry matter (TDM) of emerged seedlings of wheat and weeds in the *in vitro* experiment, as affected by the factors Species (S) (**A, B and C**) and Extract (E) (**D, E and F**). W, wheat; Alo: *A. myosuroides*; Lol: *L. multiflorum*; E_0: distilled water or control; E_B: water extracts from bergamot peel powder, E_P: water extracts from pomegranate peel powder; E_{EP5}: water extracts from EP5 commercial powder. Values are means (n=4) ± standard error. Different letters above bars indicate significant differences among species and extracts ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$. Lowercase and uppercase letters refer to F test significance at $p < 0.05$ and 0.001, respectively.

Supplementary Table 3. Effect of the factors Species (S), Extract (E) and the interaction Species x Extract (S x E) on plant inhibition index (PII) and biomass accumulation inhibition (BAI) of emerged seedlings of wheat (W), *A. myosuroides* (Alo) and *L. multiflorum* (Lol), treated with distilled water (E_0 or control) and water extracts from bergamot peel powder (E_B), pomegranate peel powder (E_P) and EP5 commercial powder (E_{EP5}), in the *in vitro* experiment.

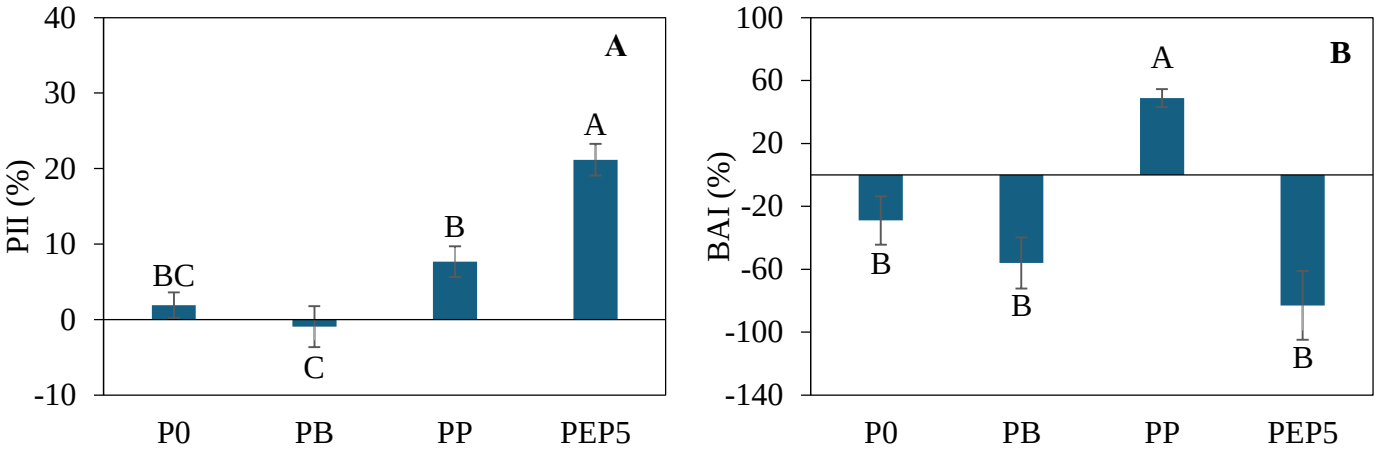
Experimental factor	PII (%)	BAI (%)
Species (S)		
W	56.0 ± 10.0 b	72.8 ± 10.9 a
Alo	75.0 ± 11.2 a	75.0 ± 11.2 a
Lol	63.8 ± 10.4 b	73.0 ± 11.0 a
Extract (E)		
E_0	0.0 ± 0.0 c	0.00 ± 0.0 b
E_B	74.1 ± 7.9 b	96.3 ± 2.5 a
E_P	85.9 ± 5.1 b	98.2 ± 1.5 a
E_{EP5}	99.6 ± 0.3 a	99.9 ± 0.0 a
Species x Extract (S x E)		
W-E_0	0.0 ± 0.0 c	0.0 ± 0.0 a
Alo- E_0	0.0 ± 0.0 c	0.0 ± 0.0 a
Lol- E_0	0.0 ± 0.0 c	0.0 ± 0.0 a
W-E_B	51.9 ± 13.0 b	96.8 ± 2.3 a
Alo- E_B	100.0 ± 0.0 a	100.0 ± 0.0 a
Lol- E_B	70.2 ± 10.1 ab	92.0 ± 7.3 a
W-E_P	73.0 ± 9.4 ab	94.7 ± 4.6 a
Alo- E_P	100.0 ± 0.0 a	100.0 ± 0.0 a
Lol- E_P	84.8 ± 8.9 a	99.8 ± 0.1 a
W-E_{EP5}	98.9 ± 1.1 a	99.9 ± 0.1 a
Alo- E_{EP5}	100.0 ± 0.0 a	100.0 ± 0.0 a
Lol- E_{EP5}	100.0 ± 0.0 a	100.0 ± 0.0 a
Significance		
S	***	ns
E	***	***
S x E	***	ns

Values are means (n=4) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; ns, not significant.

Supplementary Table 4. Effect of the factors Species (S), Extract (E) and the interaction Species x Extract (S x E) on average root number (AVG_{RN}) and average length of individual root (AVG_{RL}) per emerged seedling of wheat (W), *A. myosuroides* (Alo) and *L. multiflorum* (Lol), treated with distilled water (E_0 or control), and water extracts from bergamot peel powder (E_B), pomegranate peel powder (E_P) and EP5 commercial powder (E_{EP5}), in the *in vitro* experiment.

Experimental factor	AVG_{RN} (n°)	(AVG_{RL}) (cm)
Species (S)		
W	4.0 ± 0.2 a	5.3 ± 0.2 a
Alo	0.8 ± 0.0 b	2.1 ± 0.2 a
Lol	1.2 ± 0.1 b	4.1 ± 0.3 a
Extract (E)		
E_0	2.8 ± 0.1 a	5.8 ± 0.2 a
E_B	3.1 ± 0.6 a	1.8 ± 0.2 b
E_P	1.3 ± 0.2 b	0.5 ± 0.1 b
E_{EP5}	0.2 ± 0.1 b	0.1 ± 0.0 b
Species x Extract (SxE)		
W-E_0	4.9 ± 0.2 a	6.4 ± 0.2 a
Alo- E_0	1.0 ± 0.0 b	2.5 ± 0.2 a
Lol- E_0	1.3 ± 0.1 b	5.1 ± 0.3 a
W-E_B	4.9 ± 0.6 a	2.0 ± 0.2 a
Alo- E_B	0.0 ± 0.0 b	0.0 ± 0.0 a
Lol- E_B	1.3 ± 0.4 b	0.6 ± 0.2 a
W-E_P	1.7 ± 0.2 b	0.7 ± 0.1 a
Alo- E_P	0.0 ± 0.0 b	0.0 ± 0.0 a
Lol- E_P	0.8 ± 0.1 b	0.2 ± 0.1 a
W-E_{EP5}	0.5 ± 0.2 b	0.2 ± 0.1 a
Alo- E_{EP5}	0.0 ± 0.0 b	0.0 ± 0.0 a
Lol- E_{EP5}	0.0 ± 0.0 b	0.0 ± 0.0 a
Significance		
S	***	ns
E	***	***
SxE	***	ns

Values are means (n=4) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; ns, not significant.



Supplementary Figure 3. Plant inhibition index (PII) (**A**) and biomass accumulation inhibition (BAI) (**B**) of wheat in the first *in vivo* experiment, as affected by the factor Powder (P). P_0: untreated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

Supplementary Table 5. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on the final germination percentage (FGP), mean germination time (MGT), germination index (GI) and seed vigor index (SVI) of the two weeds, *A. myosuroides* (W_{Alo}) and *L. multiflorum* (W_{Lol}), grown on soil un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the first *in vivo* experiment.

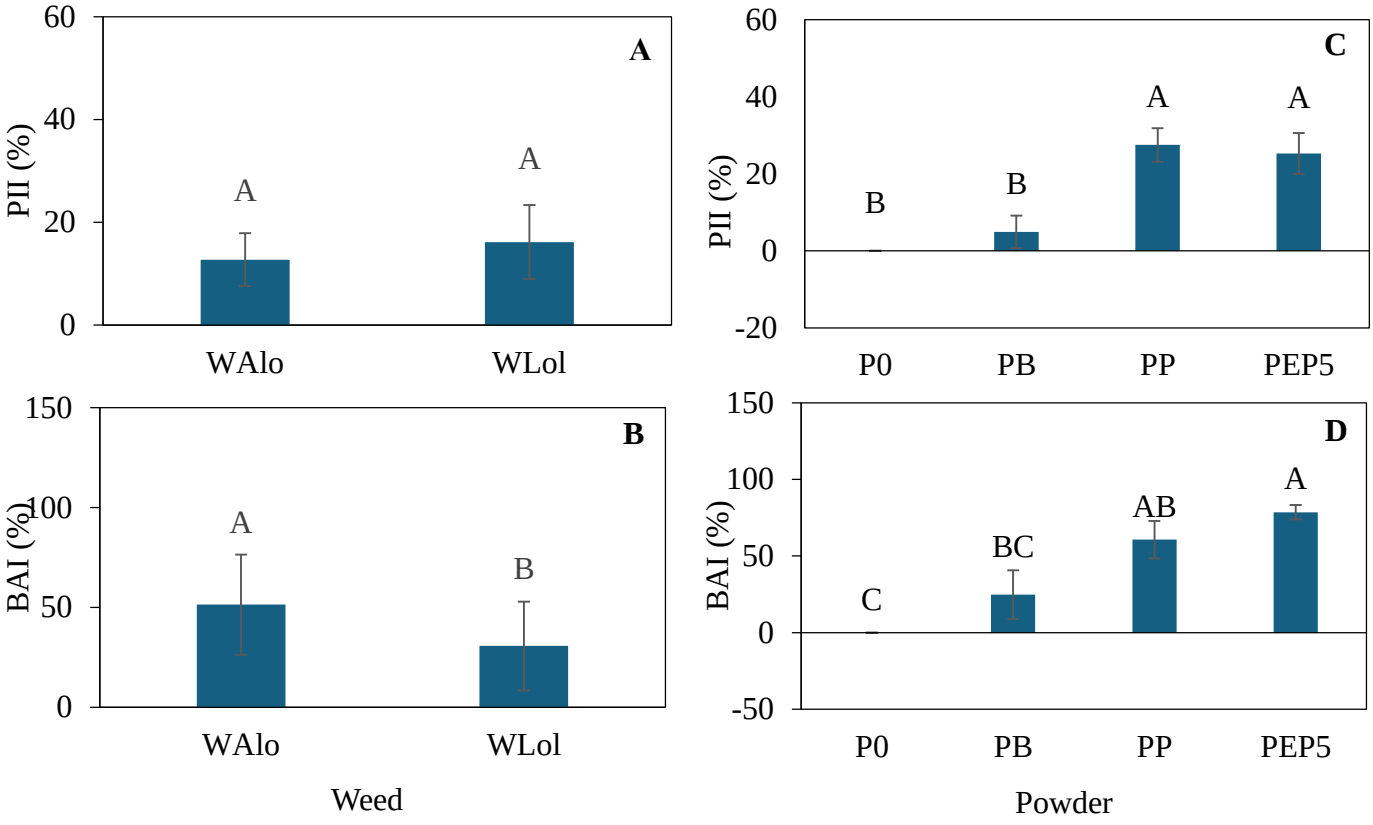
Experimental factor	FGP (%)	MGT (days)	GI	SVI
Weed (W)				
W_{Alo}	24.8 ± 2.4 b	19.7 ± 0.6 a	0.5 ± 0.4 b	700.6 ± 94.0 b
W_{Lol}	47.7 ± 3.1 a	20.9 ± 0.8 a	0.8 ± 0.6 a	1215.7 ± 84.9 a
Powder (P)				
P_0	58.9 ± 2.7 a	14.4 ± 0.3 c	1.3 ± 0.1 a	1210.1 ± 74.6 a
P_B	35.9 ± 3.7 b	20.9 ± 0.6 b	0.5 ± 0.1 b	734.0 ± 86.4 b
P_P	38.2 ± 4.5 b	22.1 ± 0.6 ab	0.5 ± 0.1 b	551.2 ± 68.2 b
P_{EP5}	12.1 ± 1.5 c	23.9 ± 1.4 a	0.2 ± 0.0 c	1337.4 ± 206.1 a
Weed × Powder (W x P)				
W_{Alo}-P_0	52.7 ± 2.7 a	15.4 ± 0.4 a	1.1 ± 0.1 b	928.5 ± 48.8 a
W_{Lol}-P_0	65.0 ± 4.2 a	13.3 ± 0.5 a	1.6 ± 0.1 a	1491.7 ± 100.3 a
W_{Alo}-P_B	18.8 ± 2.8 b	19.6 ± 0.8 a	0.3 ± 0.0 d	319.1 ± 43.5 a
W_{Lol}-P_B	53.1 ± 3.1 a	22.2 ± 0.9 a	0.7 ± 0.1 c	1148.8 ± 77.6 a
W_{Alo}-P_P	16.0 ± 2.0 b	21.3 ± 1.1 a	0.2 ± 0.0 d	233.9 ± 39.4 a
W_{Lol}-P_P	60.4 ± 3.5 a	23.0 ± 0.6 a	0.8 ± 0.1 c	868.5 ± 65.1 a
W_{Alo}-P_{EP5}	11.9 ± 1.8 b	22.6 ± 1.7 a	0.2 ± 0.0 d	1321.1 ± 299.0 a
W_{Lol}-P_{EP5}	12.3 ± 2.3 b	25.2 ± 2.1 a	0.2 ± 0.0 d	1353.7 ± 293.4 a
Significance				
W	.***	ns	***	***
P	***	***	***	***
W x P	***	ns	***	ns

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; ns, not significant.

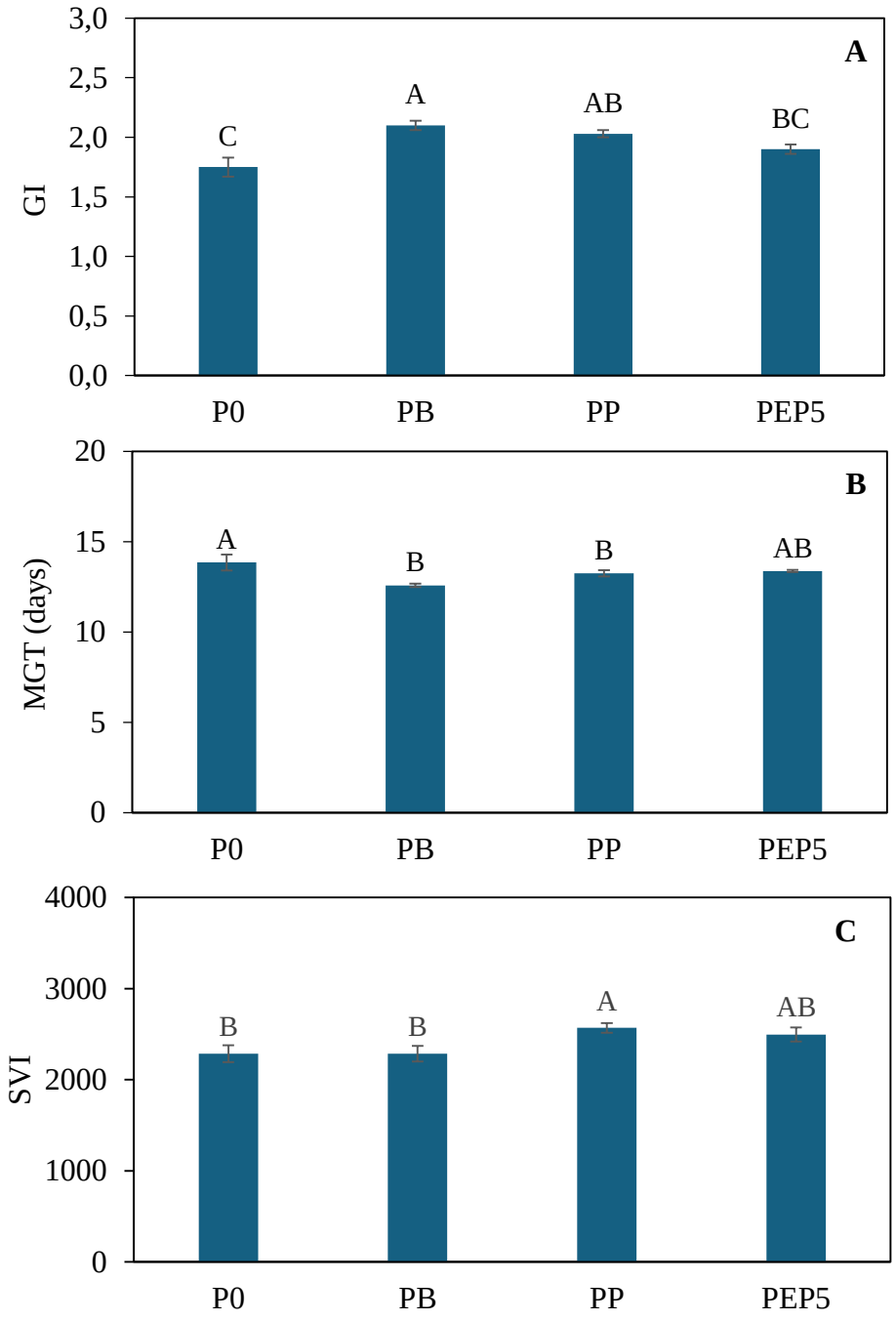
Supplementary Table 6. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) **on** the root (RL), shoot (SL) and total length (TL), root (RDM), shoot (SDM) and total dry matter content (TDM) of the two weeds, *A. myosuroides* (W_{Alo}) and *L. multiflorum* (W_{Lol}), grown on soil un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the first *in vivo* experiment.

Experimental factor	RL	SL	TL	RDM	SDM	TDM
	(cm)			(% fw)		
Weed (W)						
W_{Alo}	4.2 ± 0.1 b	12.3 ± 0.2 b	16.6 ± 0.2 b	32.0 ± 7.9 a	22.8 ± 7.3 a	24.0 ± 7.4 a
W_{Lol}	5.1 ± 0.2 a	14.5 ± 0.3 a	19.7 ± 0.4 a	31.4 ± 8.2 a	20.1 ± 5.0 b	20.9 ± 5.2 b
Powder (P)						
P_0	5.6 ± 0.1 a	15.0 ± 0.2 a	20.6 ± 0.3 a	27.3 ± 0.7 c	18.3 ± 0.4 b	19.0 ± 0.4 c
P_B	4.5 ± 0.2 b	15.7 ± 0.3 a	20.3 ± 0.4 a	30.5 ± 1.3 bc	19.9 ± 1.1 b	20.6 ± 1.1 bc
P_P	3.9 ± 0.2 b	10.5 ± 0.2 c	14.4 ± 0.3 c	32.5 ± 0.9 ab	21.5 ± 1.0 b	23.2 ± 1.0 b
P_{EP5}	4.6 ± 0.3 b	12.6 ± 0.5 b	17.1 ± 0.7 b	36.5 ± 2.0 a	26.1 ± 1.3 a	27.2 ± 1.4 a
Weed x Powder (W x P)						
W_{Alo}-P_0	4.4 ± 0.2 bc	13.2 ± 0.2 b	17.6 ± 0.3 b	29.1 ± 0.8 a	18.9 ± 0.5 cd	19.6 ± 0.5 c
W_{Lol}-P_0	6.6 ± 0.2 a	16.4 ± 0.2 a	23.0 ± 0.3 a	25.5 ± 0.9 a	17.7 ± 0.6 d	18.3 ± 0.6 c
W_{Alo}-P_B	3.6 ± 0.3 c	13.5 ± 0.4 b	17.1 ± 0.5 bc	30.5 ± 2.5 a	19.7 ± 2.1 b-d	20.5 ± 2.1 bc
W_{Lol}-P_B	4.9 ± 0.2 b	16.6 ± 0.3 a	21.5 ± 0.5 a	30.5 ± 0.8 a	20.1 ± 0.6 b-d	20.8 ± 0.6 bc
W_{Alo}-P_P	4.3 ± 0.4 bc	10.2 ± 0.5 c	14.5 ± 0.7 cd	32.8 ± 1.3 a	25.0 ± 1.3 ab	27.4 ± 1.1 a
W_{Lol}-P_P	3.8 ± 0.2 c	10.5 ± 0.3 c	14.3 ± 0.3 d	32.2 ± 1.4 a	18.0 ± 0.9 d	18.9 ± 0.9 c
W_{Alo}-P_{EP5}	4.4 ± 0.3 bc	9.3 ± 0.5 c	13.6 ± 0.7 d	35.5 ± 2.5 a	27.5 ± 2.1 a	28.6 ± 2.1 a
W_{Lol}-P_{EP5}	4.8 ± 0.4 bc	15.7 ± 0.7 a	20.4 ± 1.1 a	37.4 ± 3.1 a	24.8 ± 1.7 a-c	25.7 ± 1.8 ab
Significance						
W	***	***	***	ns	*	*
P	***	***	***	***	***	***
W x P	***	***	***	ns	*	*

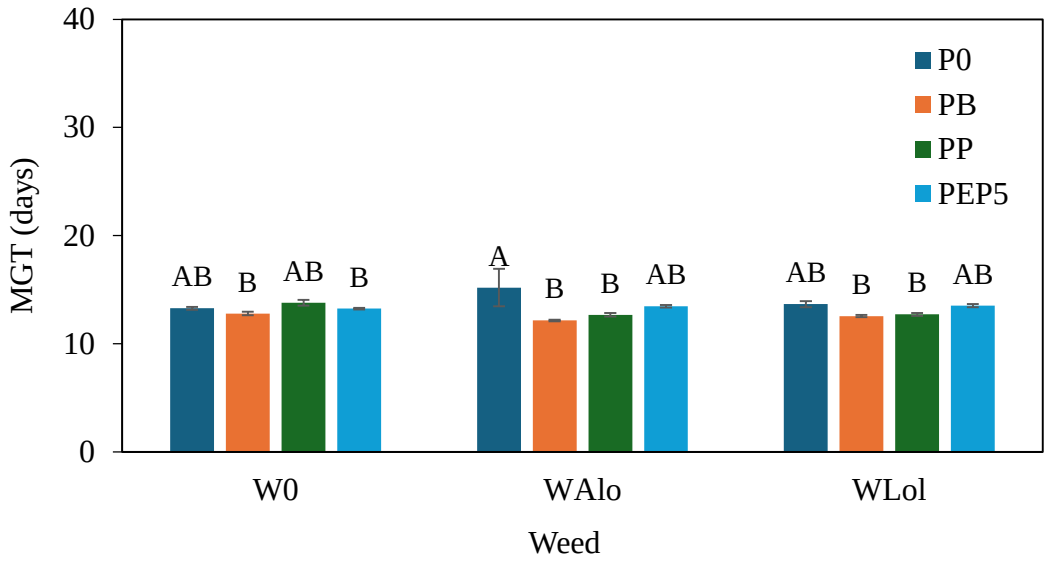
Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.



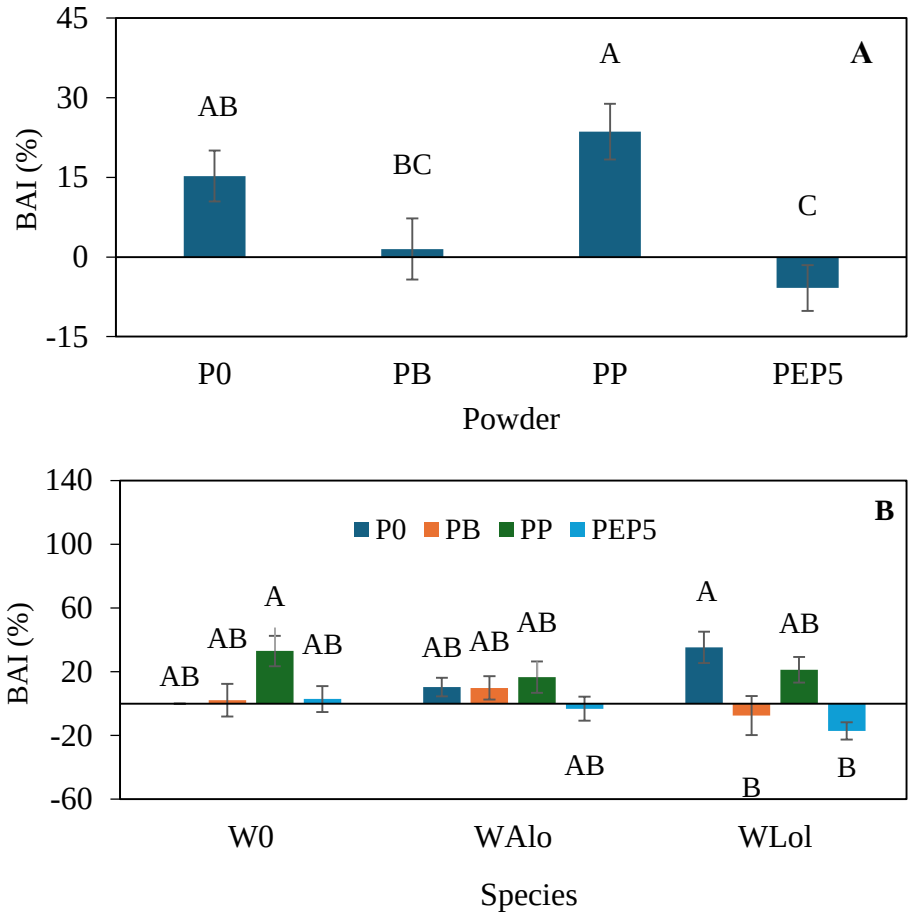
Supplementary Figure 4. Plant inhibition index (PII) and biomass accumulation inhibition (BAI) of weeds in the first *in vivo* experiment, as affected by the factors Weed (W) (**A and B**) and Powder (P) (**C and D**). W_{Lol}: *A. myosuroides*; W_{Alo}: *L. multiflorum*. P_0: un-treated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.



Supplementary Figure 5. Germination index (GI) (**A**), mean germination time (MGT) (**B**) and seed vigor index (SVI) (**C**) of wheat in the second *in vivo* experiment, as affected by the factor Powder (P). P_0: un-treated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.



Supplementary Figure 6. Mean germination time (MGT) of wheat in the second *in vivo* experiment, as affected by the interaction Weed x Powder (W x P). W_0: weed-free soil or control; W_{Alo}: soil infested with *A. myosuroides*; W_{Lol}: soil infested with *L. multiflorum*; P_0: un-treated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

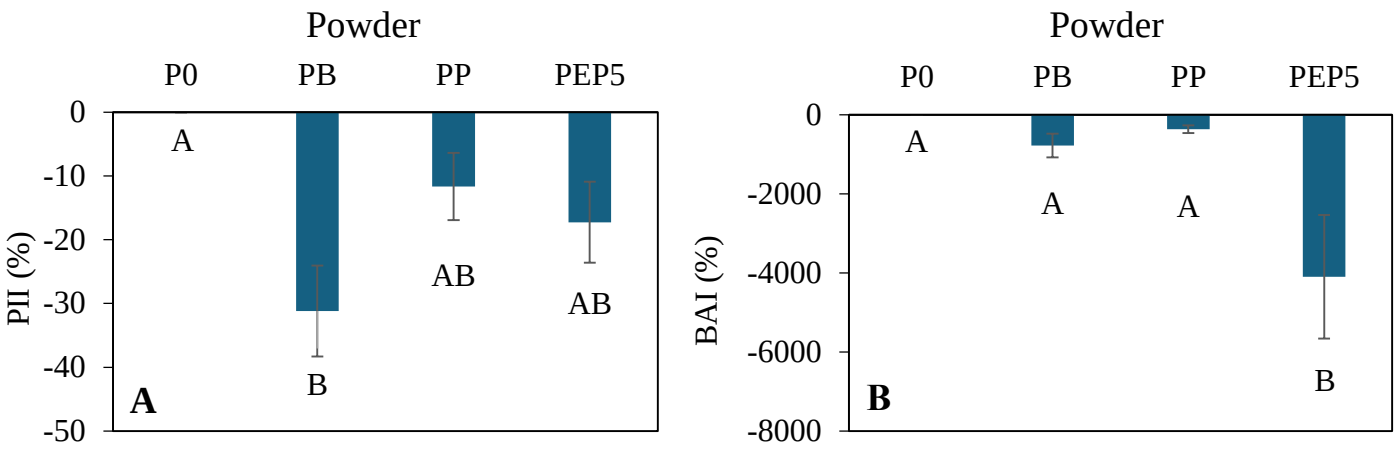


Supplementary Figure 7. Biomass accumulation inhibition (BAI) of wheat in the second *in vivo* experiment, as affected by the factor Powder (P) (**A**) and the interaction Weed x Powder (W x P) (**B**). W_0: weed-free soil or control; W_{Alo}: soil infested with *A. myosuroides*; W_{Lol}: soil infested with *L. multiflorum*; P_0: un-treated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

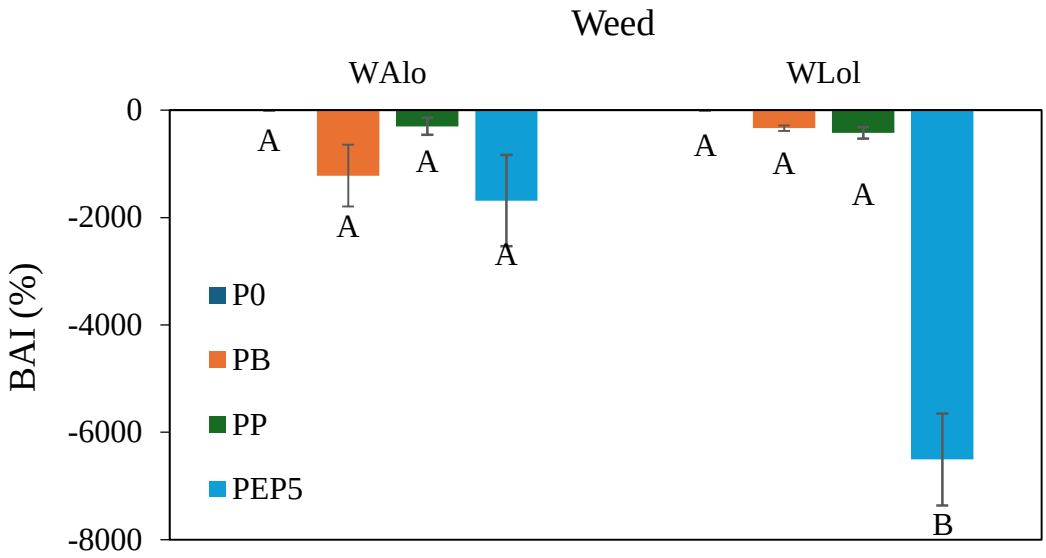
Supplementary Table 7. Effect of the factors Weed (W), Powder(P) and the interaction Weed x Powder (W x P) on the root (RL), shoot (SL) and total length (TL), root (RDM), shoot (SDM) and total dry matter content (TDM) of the two weeds, *A. myosuroides* (W_{Alo}) and *L. multiflorum* (W_{Lol}), grown on soil un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the second *in vivo* experiment.

Experimental factor	RL	SL	TL	RDM	SDM	TDM
	(cm)			(%)		
Weed (W)						
W_{Alo}	8.1 ± 0.2 a	11.6 ± 0.2 b	19.7 ± 0.4 b	27.5 ± 2.4 a	19.4 ± 2.0 a	20.2 ± 1.4 a
W_{Lol}	8.3 ± 0.1 a	16.7 ± 0.3 a	25.0 ± 0.3 a	30.4 ± 2.7 a	21.8 ± 1.7 a	21.8 ± 1.5 a
Powder (P)						
P_0	8.1 ± 0.2 ab	12.5 ± 0.4 d	20.6 ± 0.5 c	31.2 ± 4.1 ab	23.1 ± 3.3 a	24.2 ± 2.6 a
P_B	8.6 ± 0.3 a	17.6 ± 0.3 a	26.2 ± 0.5 a	25.8 ± 3.3 b	19.1 ± 3.2 a	17.6 ± 1.5 b
P_P	7.7 ± 0.2 b	15.5 ± 0.4 b	23.2 ± 0.5 b	20.3 ± 1.4 b	17.1 ± 1.3 a	17.6 ± 1.1 b
P_{EP5}	8.6 ± 0.2 a	14.9 ± 0.4 c	23.4 ± 0.6 b	38.3 ± 3.7 a	23.0 ± 2.0 a	24.5 ± 2.0 a
Weed x Powder (W x P)						
W_{Alo}-P_0	8.5 ± 0.5 a	9.4 ± 0.4 a	17.9 ± 0.8 a	32.4 ± 6.4 a	16.0 ± 2.9 a	18.2 ± 2.3 bc
W_{Lol}-P_0	8.0 ± 0.3 a	13.4 ± 0.4 a	21.4 ± 0.5 a	30.0 ± 5.5 a	30.3 ± 4.9 a	30.3 ± 3.7 a
W_{Alo}-P_B	8.3 ± 0.5 a	13.6 ± 0.4 a	22.0 ± 0.7 a	19.1 ± 1.8 a	20.9 ± 6.4 a	17.9 ± 2.9 bc
W_{Lol}-P_B	8.7 ± 0.3 a	19.0 ± 0.4 a	27.8 ± 0.6 a	32.5 ± 5.7 a	17.4 ± 1.2 a	17.4 ± 1.2 bc
W_{Alo}-P_P	7.3 ± 0.3 a	11.8 ± 0.3 a	19.1 ± 0.5 a	19.7 ± 1.4 a	15.9 ± 1.7 a	16.9 ± 1.1 c
W_{Lol}-P_P	7.9 ± 0.2 a	17.8 ± 0.5 a	25.7 ± 0.6 a	20.9 ± 2.5 a	18.4 ± 1.9 a	18.4 ± 1.9 bc
W_{Alo}-P_{EP5}	8.9 ± 0.5 a	10.8 ± 0.5 a	19.6 ± 0.9 a	38.7 ± 4.4 a	24.9 ± 3.1 a	28.0 ± 2.9 ab
W_{Lol}-P_{EP5}	8.5 ± 0.2 a	16.1 ± 0.5 a	24.6 ± 0.6 a	38.0 ± 6.1 a	21.0 ± 2.4 a	21.0 ± 2.4 a-c
Significance						
W	ns	***	***	ns	ns	ns
P	**	***	***	**	ns	**
W x P	ns	ns	ns	ns	ns	**

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.



Supplementary Figure 8. Plant inhibition index (PII) (**A**) and biomass accumulation inhibition (BAI) (**B**) of weeds in the second *in vivo* experiment, as affected by the factor powder (P). P_0: un-treated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. P_0: powder-free treatment (control); P_B: bergamot powder; P_P: pomegranate powder; P_{EP5}: commercial EP5 powder. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.



Supplementary Figure 9. Biomass accumulation inhibition (BAI) of weeds in the second *in vivo* experiment, as affected by the interaction Weed x Powder (W x P). W_0: weed-free soil or control; W_{Alo}: soil infested with *A. myosuroides*; W_{Lol}: soil infested with *L. multiflorum*; P_0: un-treated soil or control; P_B: soil treated with bergamot peel powder; P_P: soil treated with pomegranate peel powder; P_{EP5}: soil treated with EP5 commercial powder. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

Objective 4. Evaluation of efficacy of bergamot, pomegranate, and EP5 against *Athelia rolfsii* (syn. *Agroathelia rolfsii*), *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* under both *in vitro* and *in vivo* conditions on tomato crop.

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The Potential of Bergamot and Pomegranate Wastes as Putative Plant-Based Antifungal Products Against Soilborne Pathogens of Tomato: Preliminary Experiments

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Abstract

Traditional disease management, which is based on the application of synthetic chemical products, has negatively affected human health and the environment. A sustainable approach based on the application of natural compounds and microorganisms is potentially better for consumer health. Thus, the aim of this study was to evaluate the efficacy of plant-based and/or organic products against soilborne fungal pathogens of tomato. A preliminary in vitro experiment was performed to select potential putative inhibitory products (PIPs) and fungal pathogens that were then used in an in vivo experiment conducted inside a greenhouse that mimics real-world field conditions. For the greenhouse experiment, bergamot and pomegranate wastes and the commercial product EP5 were selected as the PIPs to control *Agroathelia rolfsii*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* growth. Each pot was artificially inoculated three days before the low-dose treatment, and one tomato seedling was transplanted into each pot four days after the treatment. Data regarding the phytosanitary status of the plants and roots, as well as their length and weight, were collected after 45 days, and the results obtained demonstrate that plant-derived products were able to mitigate fungal diseases, with pomegranate waste being the most effective. Also, the EP5 product, as a resistant inducer, was able to significantly improve the natural defense of tomato plants, resulting in it being the best PIP used. Mycological analyses were performed on the roots to assess the presence of inoculated fungal pathogens after natural product treatment. Overall, the results confirm that the PIPs are suitable for crop management, but the outcomes are variable. In general, pomegranate waste and EP5 significantly protected the roots against fungal attacks, while bergamot waste showed lower efficacy. This trend was not observed for plant length and weight, as the treated plants showed results similar to those of the untreated controls. In conclusion, natural products are a valid alternative to chemicals, as they demonstrate both efficacy and safety, but their potential should be further investigated in field trials.

Keywords: plant-based antifungal product; soilborne fungi; crop protection; inhibition; tomato crop

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1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and consumed agricultural food commodities, with notable benefit to human health [1]. In 2023, global tomato production reached the highest record, accounting for 192 million tonnes [2]. The

largest producer is China, followed by India, the United States, Turkey, and European countries. Among European countries, Italy is the leading producer, accounting for 36% (approximately 6.0 million tonnes) of the total production in 2024, followed by Spain (27%) and Portugal (10%) [3]. Among Italian regions, Apulia remains the major contributor, accounting for 22.3% of the nation's production, of which 19.4% comes from the Foggia province as the primary hub [4].

Given the global importance of tomato, its health status is also of global interest, as tomato yields are consistently threatened by microbial diseases, with fungi and oomycetes being the major causal agents [5]. *Alternaria solani*, *Botrytis cinerea*, and *Phytophthora infestans* represent the major aerial pathogens that attack the aerial parts of the tomato plant [6]. In particular, *A. solani* causes seedling blight, fruit rot, and severe leaf rust, leading to complete defoliation [7]. *B. cinerea* causes necrotic-enzyme-induced soft rot via cell wall degradation [8], while *P. infestans* is responsible for shoot and leaf necrosis, fruit rot, and rapid plant death [5]. Significant threats also are caused by several soilborne pathogens, among which *Agroathelia rolfsii*, *Fusarium oxysporum* f.sp. *lycopersici*, and *Sclerotinia sclerotiorum* are the most widespread in soils worldwide [9]. *A. rolfsii* mainly causes stem and collar rot, but it can also induce fruit rot when in contact with the soil [10]. *F. oxysporum*, as a vascular pathogen, causes root colonization, which leads to wilting and plant death [11]. *S. sclerotiorum* is particularly destructive and causes severe root rot and water-soaked lesions at the stem joints, potentially resulting in yield losses exceeding 50% [12,13]. Chemical control remains the primary and effective means for reducing the destructive potential of fungal pathogens in agriculture; however, in addition to the high costs and the risk of resistance development, their use is increasingly restricted due to their detrimental effects on human health and the environment [6]. Therefore, chemical pesticides are responsible for widespread negative impacts, as sprayed pesticides can enter the human bloodstream through inhalation or skin contact, causing acute symptoms such as dermatitis, respiratory irritation, nausea, and headaches [14]. Moreover, chronic exposure to pesticide residues in food is often associated with severe human diseases, including cancer, Alzheimer's disease, and diabetes mellitus, which often occur due to DNA damage [14]. Chemical pesticides also negatively affect the environment, as toxic residues can persist in the soil, harm beneficial organisms (suppressive, antagonistic, etc.), and compromise soil fertility [15–17]. Chemical residues are frequently detected in various food and beverage products [18]. To address these concerns, the European Parliament has enforced stringent regulations by limiting maximum residue levels (MRLs) in food and banning the use of specific active fungicides in open fields since the 1980s [19,20]. These regulatory measures have stimulated scientific research to investigate the effectiveness of natural alternatives, including agricultural wastes (AWs) and biological control agents (BCAs). Several studies confirm that BCAs, such as *Trichoderma* [21–25] and *Bacillus* spp. [26–28], are effective for controlling fungal pathogens. Beyond microorganisms, plant products and their extracts can also offer a viable alternative to chemicals. Indeed, extracts from *Azadirachta indica*, *Cannabis sativa*, *Cassia fistula*, *Cinnamomum verum*, and *Lantana camara* display high inhibitory activity against multiple fungal pathogens [29–31]. Morea et al. [32] demonstrated that plant powders from numerous botanical species were able to inhibit the growth of more than 30 different fungal pathogens, and other studies have reported significant results regarding the pharmacological and antifungal activities of pomegranate and bergamot extracts, which are attributed to their rich metabolic profiles [33–36]. Carlucci et al. [37] and Morea et al. [32] assayed EP5 in their experiments to confirm its ability to protect grape berries against *Botrytis cinerea* infections in postharvest conditions and to compare its antifungal activity against more than 30 severe phytopathogens under in vitro conditions. Based on previous research on the efficacy of plant-derived products in controlling severe microbial diseases

in plants, their potential contribution to the circular economy in agriculture, and their very low impact on humans and the environment, the aim of the present study is to assess the efficacy of putative plant-based wastes against soilborne fungal pathogens of tomato crops. These natural putative fungicides, as minimally processed products, are tested to ensure that they can be used in organic agriculture, without any expensive and non-eco-friendly chemical transformation. Therefore, the objectives of this study are to conduct (a) an in vitro assessment of plant products, including those from laurel, eucalyptus, pomegranate, sage, bergamot, and false pepper, and a commercial organic product against ten soilborne fungal pathogens; (b) a greenhouse-based evaluation of low doses of the selected products to assess their efficacy to protect tomato roots against fungal pathogens; and (c) an assessment of the agronomic quality indices of tomato plant growth after treatment with putative plant-based antifungal products.

2. Materials and Methods

2.1. Preparation of Putative Inhibitory Products

The putative inhibitory products (PIPs) were prepared according to the method described by Morea et al. [32]. Different plant products with potential antifungal activity were collected from two botanical species: pomegranate (*Punica granatum* L.; peel) (Pg) from open-field agricultural crops in Foggia province (Apulia region, Italy) and bergamot-derived (*Citrus bergamia* Risso & Poit.; peel) (Cb) from agro-industrial waste obtained after essential oil extraction in Gioia Tauro-origin (Calabria, Italy). After collection, the plant tissues were carefully washed, cut into small pieces, and left to dry at room temperature until the weight was reduced by approximately 50%. The semi-dehydrated plant tissues were placed on a stove at 30 °C (FN 500 Sterilizer, Nuve, Ankara, Turkey) until constant weight was achieved. The plant PIPs were ground as finely as possible using an electric mill equipped with a fine-mesh screen (Geomil-50P) at 600 nm to obtain plant powders, which were then stored in plastic jars in darkness. In addition, a commercial product (EP5-Protect) (EP5) that is used as resistance inducer in organic agriculture was tested, whose composition consists of minerals and silicates [bentonite (30%), citric acid (5%), iron (10%), zinc (6%), and manganese (6.5%) sulphate; Phenom Biotech srl, Milan Italy].

2.2. Soilborne Fungal Phytopathogens

To assess the ability of the PIPs to inhibit fungal growth, three fungal species considered as severe soilborne pathogens, namely *Agroathelia rolfsii*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*, were used; these fungi were obtained from the fungal collection of the Laboratory of Plant Pathology and Diagnostic of the Department of Agricultural, Food, Natural Resources and Engineering Sciences (DAFNE) of the University of Foggia, Italy. The taxonomic identities of these fungi, which were determined via morphological and molecular tools, have been reported by Morea et al. [32] using the DNA extraction protocol described by Carlucci et al. [38].

2.3. Assessment of Putative Inhibitory Products (PIPs) Against Fungal Pathogens Under In Vitro Conditions

Each plant powder and EP5 were used at a concentration of 2% (w:v), and added to the PDA medium (39 g/L; Sigma-Aldrich, Milan, Italy). After adjusting the pH to 6.0, the latter was thermally treated at 115 °C for 15 min in an autoclave. Inhibitory activity was assessed on 90 mm Petri dishes containing the PDA medium. Fungal plugs of 4 mm in diameter, which were taken from the margins of 14-day-old colonies of each fungal pathogen, were placed at the center of 90 mm Petri dishes containing the PDA medium supplemented with PIPs and incubated in darkness at 21 ± 3 °C for up to 21 days, depending on the mycelial

growth of each fungal species, while PDA plates without PIPs were used as controls, as performed by Morea et al. [32]. The experiment was replicated three times, and mycelial growth was recorded every 48 h by measuring two perpendicular diameters. According to Carlucci et al. [39] and Morea et al. [32], inhibitory activity (IA; %) was calculated as the percentage of mycelium growth inhibition compared to the control using the formula:

$$IA = \left(\frac{D_1 - D_2}{D_1} \right) \times 100$$

where D_1 and D_2 are the measured perpendicular diameters of the fungal colonies on PDA control plates and PDA plates amended with PIP powders, respectively.

Fungal growth was modeled according to Bevilacqua et al. [40], who used a logistic equation modified by Dantigny et al. [41], and it is determined using following formula:

$$\frac{D_{max}}{1 + exp[k(\tau - t)]}$$

where D_{max} is the maximum diameter of the fungal colony (55 mm); k is the rate of fungal growth (mm hours^{-1}); τ is the time to attain half of D_{max} (hours); and t is the time (hours).

This Dantigny function utilizes three fitting parameters, i.e., the rate of fungal growth, the maximum extent of germination, and the time to attain half of the maximal density/probability of germination. Thus, the modified equation, which was based on Bevilacqua et al. [40], was used to model the radial increase in the diameter of fungal colonies on the plates. Moreover, the maximum extent of fungal growth was set to 55 mm (i.e., the diameter of the plates). The fitting procedure was intended to estimate the rate of fungal growth, reported as diameter increase per day, and the parameter τ, which refers to the time (hours) needed to attain a diameter of 27.5 mm on the plates. The Dantigny equation satisfactorily fitted all the data sets, with regression coefficients ranging from 0.909 to 0.999.

2.4. Evaluation of PIPs to Control Fungal Pathogens Under In Vivo Conditions

2.4.1. Artificial Fungal Inoculum Preparation

The artificial inoculum of each fungal pathogen was prepared by infecting 100 g of wheat kernels (RGT Voilur variety; Semetica, Bologna, Italy) preliminarily soaked in tap water in a 500 mL glass jar for 12 h. Then, the water was removed, and the kernels were sterilized three times at 121 °C for 20 min. The sterilized kernels were then inoculated with 5 mL of the fungal propagule suspension at a concentration of $1 \cdot 10^5$ cfu mL^{-1}, which was obtained by scraping the surface of a 14-day-old fungal colony with a scalpel and collecting the propagules in a sterile solution (distilled H_2O with 0.5% Tween 20). All inoculated jars were sealed with parafilm and incubated in the dark at 25 °C ± 3 °C for 21 days.

2.4.2. Setup of the Greenhouse Experiment

The experiment was conducted in a greenhouse that mimics real-world field conditions from May to July 2024 at the Department DAFNE of the University of Foggia. For this experiment, two layers of non-woven fabric were placed at the bottom of 1.5 L pots to prevent soil loss and covered with 5 cm of commercial Gaia soil (Agrochimica S.p.A., Bolzano, Italy). Twelve kernels artificially inoculated with each fungal pathogen were placed equidistantly from each other on the moistened soil surface and covered with approximately 1 cm of soil. The soil was then watered to promote fungal growth and covered with a plastic film. After 72 h, 3 g aliquots of powder from each PIP were placed on the soil surface, covered with another 1 cm of soil, and again covered with a plastic film. Finally, after an additional 72 h, one tomato seedling was placed at the center of the pot and

covered with 1 cm of soil. The experiment consisted of 16 different trials with 24 replicates. Un-inoculated and un-treated pots were used as negative controls (Table 1).

Table 1. PIP and fungal composition of each trial under different settings in the greenhouse experiment.

PIP	Fungus	Id Trial
-	-	Un-treated
-	*Agroathelia rolfsii*	*A. rolfsii*
-	*Fusarium oxysporum*	*F. oxysporum*
-	*Sclerotinia sclerotiorum*	*S. sclerotiorum*
Citrus bergamia	-	*C. bergamia*
EP5	-	EP5
Punica granatum	-	*P. granatum*
Citrus bergamia	*Agroathelia rolfsii*	*C. bergamia-A. rolfsii*
Citrus bergamia	*Fusarium oxysporum*	*C. bergamia-F. oxysporum*
Citrus bergamia	*Sclerotinia sclerotiorum*	*C. bergamia-S. sclerotiorum*
EP5	*Agroathelia rolfsii*	EP5-*A. rolfsii*
EP5	*Fusarium oxysporum*	EP5-*F. oxysporum*
EP5	*Sclerotinia sclerotiorum*	EP5-*S. sclerotiorum*
Punica granatum	*Agroathelia rolfsii*	*P. granatum-A. rolfsii*
Punica granatum	*Fusarium oxysporum*	*P. granatum-F. oxysporum*
Punica granatum	*Sclerotinia sclerotiorum*	*P. granatum-S. sclerotiorum*

During the greenhouse experiment, the tomato plants received 150–200 mL of water, and no fertilization was carried out. After 45 days, to assess damage caused by the diseases, the tomato plants were subjected to phytosanitary status determination by observing the symptoms that occurred on the aerial plant parts using an empirical six-class pathometric scale (0 = healthy; 1 = slightly yellow; 2 = completely yellow; 3 = slightly necrotized; 4 = completely necrotized; and 5 = dead). The plants were then gently removed from the pots, and the roots were carefully washed and assessed with a seven-class empirical pathometric scale (0 = no symptoms observed; 1 = 1–15%; 2 = 16–35%; 3 = 36–50%; 4 = 51–70%; 5 = 71–85%; and 6 = 86–100%). However, to evaluate phytosanitary status based on the symptoms that occurred on the collar, a different six-class empirical pathometric scale (0 = healthy; 1 = hydropic tissues; 2 = brown; 3 = slightly rotten; 4 = rotten; and 5 = heavily rotten) was used. The phytosanitary status of the aerial plant part, root, and collar was expressed as disease incidence according to the McKinney index (MkI; %) [42], which was determined according to the following formula:

$$MkI\ (\%) = \frac{\sum(N_n \times C_n)}{N_{tot} \times C_{max}} \times 100$$

where N_n is the number of cases observed for each class, C_n is the reference class, N_{tot} is the total number of cases observed, and C_{max} is the highest class of the scale.

Moreover, the plant length (including root) of young tomato plants that survived was monitored once a week, and at the end of each plant's lifecycle, compactness (g cm^{-1}) was assessed as a growth index according to Nam et al.'s study [43], as it is generally known that when plants undergo drought stress, stem length decreases and compactness increases [44]. Therefore, compactness (growth index) was determined using the following formula [43]:

$$Compactness = \frac{Dry\ weight\ (g)}{Plant\ length\ (cm)} \times 100$$

2.5. Re-Isolation of Artificially Inoculated Fungal Pathogens

Then, the tomato roots were subjected to mycological analyses to determine the presence of the fungi inoculated (re-isolation frequency (RIF; %)), and RIF was calculated according to the following formula:

$$RIF\ (\%) = \frac{N^{\circ}\ isolated\ colonies}{Total\ n^{\circ}\ of\ isolations} \times 100$$

The roots from each plant were gently washed with distilled water to remove soil and cut into approximately 1.0 cm pieces, and the surface of the tissue pieces was superficially disinfected according to the method described by Fisher et al. [45]. For each trial, six root pieces were placed on the surface of PDA with 500 ppm of streptomycin (Sigma-Aldrich, Milan, Italy), and two plates were prepared for each trial. The fungal colonies grown after 21 days were subjected to morphological and microscopic observations to ascertain the identity of the fungi artificially inoculated.

2.6. Statistical Analyses

Statistical analyses were performed on the datasets collected for all the experiments (inhibition, bio-fumigation, and PIP activity persistence) using the JMP software package, version 16.2 (SAS Institute Inc., Cary, NC, USA). The percentage data of both PIP activities, namely inhibition of fungal growth on the PDA plate (IA) and control of fungal disease by artificial fungal inoculation (MkI), were arcsine root-square-transformed in Excel 2007 using the formula DEGREES(ASIN(SQRT(X))), where X is the percentage value.

To test if the in vitro and in vivo datasets followed normal distributions, the Shapiro–Wilk test (W test) was applied. Homogeneity of variance was assessed using the Levene test based on the number of fungal species (three in the in vitro experiment; three in the in vivo experiment) and the number of PIPs (three in the in vitro experiment; three in the in vivo experiment). A factorial ANOVA analysis was performed to determine the statistical significance of IA and the MkI by PIP against fungal growth under in vitro and in vivo conditions, as well as differences due to the sensitivity of each fungus assayed, and to detect any interaction between these factors (fungus × PIP). One-way ANOVA was performed to determine significant differences in inhibiting activity (IA) caused by each fungal species tested, as well as any difference due to the three PIPs used under in vitro conditions; disease incidence (MkI) by PIP and fungus under in vivo conditions was also analyzed. Tukey's tests were used to compare treatment means at a significance level of $p < 0.01$. Plant length and dry matter were also statistically analyzed using one-way and factorial ANOVAs, considering fungal pathogens and PIPs, using JMP version 16.2 (SAS Institute Inc., Cary, NC, USA). Regarding the kinetics of fungal pathogens, curve fitting was performed through the software Statistica for Windows version 12.0; (Statsoft, Tulsa, OK, USA) using the least square approach. The experimental data were collected for 18 months from January 2024 to August 2025.

3. Results

3.1. Effectiveness of Putative Inhibitory Products (PIPs) Against Fungal Pathogens Under In Vitro Conditions

Figure 1 (as well as Tables S1–S3) presents the results of a comprehensive scientific analysis of the effectiveness of specific natural or mineral products (PIPs) in inhibiting fungal growth. This analysis was conducted under in vitro conditions to assess the preliminary inhibitory activity of natural products before testing them under in vivo conditions. In particular, panel (a) shows data related to the sensitivity of three fungal pathogens when exposed to the PIPs, highlighting that *A. rolfsi* was the least sensitive with an IA of 42.73%,

while *F. oxysporum* was the most sensitive with an IA of 68.94%, and *S. sclerotiorum* was inhibited with an IA value of 53.03% (Table S1). Panel (b) compares the effectiveness of the PIPs against the selected fungi to determine their "relative antifungal potential". The graph shows that, among the plant products, pomegranate (IA = 43.03%) was more effective than bergamot (IA = 21.67%), but EP5, as a mineral product, demonstrated the highest efficacy in controlling all fungal pathogens, with IA values of 100.00% (Table S2). The graph in panel (c) of Figure 2 displays the interaction between specific fungal pathogens and the PIPs (EP5, pomegranate, and bergamot), showing that EP5 was the most effective treatment that inhibited all fungi, with IA values of 100.00%. In contrast, bergamot had high efficacy and specificity against *F. oxysporum* (IA = 52.27%), no efficacy and specificity against *S. sclerotiorum* (IA = 0.00%), and very low efficacy against *A. rolfsii* (IA = 12.73%) (Table S3 and Figure 2).

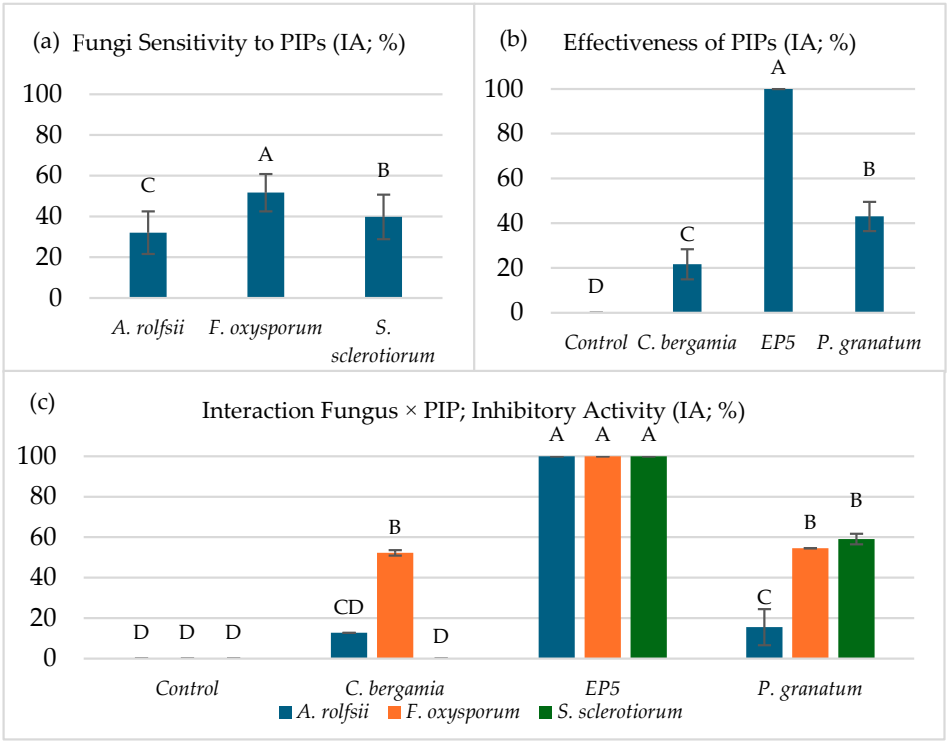


Figure 1. In vitro evaluation of fungal pathogen sensitivity and inhibition potential of putative inhibitory products (PIPs) after 21 days: (**a**) sensitivity of different fungal pathogens to PIPs, expressed as inhibition activity (IA; %); (**b**) comparative efficacy of PIPs against fungal pathogens, showing their relative antifungal potential; (**c**) interaction effects between fungal pathogens and PIPs (EP5, *P. granatum*, and *C. bergamia*), expressed as IA percentages. Data were analyzed using one-way and factorial ANOVAs and Tukey's Test ($p < 0.01$). Means with the same capital letters indicate no significant differences.

Figure 3 (Table S4) shows the growth trends of *A. rolfsii*, *F. oxysporum*, and *S. sclerotiorum* in the presence of bergamot, EP5, and pomegranate. Among the PIPs, only EP5's effect was not influenced by the presence of fungal pathogens. Among the fungal pathogens, *A. rolfsii* growth was not slowed by the PIPs. In fact, except for EP5 (τ = 480.00 h), *Bergamot* (τ = 74.80 h) and pomegranate (τ = 65.50 h) showed τ values close to those of the control (τ = 68.12 h). *F. oxysporum*'s sensitivity to the PIPs was higher than that of *A. rolfsii*. In this case, the growth kinetics of the control (τ= 72.41 h) was lower than those observed with bergamot, EP5, and pomegranate, with τ values of 480.00, 95.24, and 92.19 h, respectively.



Figure 2. Inhibitory activity (IA) of putative inhibitory products (PIPs) under in vitro (after 21 days) and in vivo (after 45 days) conditions: (**a**) IA of EP5 against *Fusarium oxysporum*; (**b**) IA of *Punica granatum* against *Sclerotinia sclerotiorum*; (**c**) IA of *Citrus bergamia* against *Agroathelia rolfsii*; (**d**) healthy roots treated with EP5 against *F. oxysporum*; (**e**) unhealthy roots treated with *P. granatum* against *S. sclerotiorum*; (**f**) severely unhealthy roots treated with *C. bergamia* against *A. rolfsii* showing significant root destruction; (**g**) healthy collar treated with EP5 against *F. oxysporum*; (**h**) unhealthy collar treated with *P. granatum* against *S. sclerotiorum* showing collar discoloration; (**i**) severely unhealthy collar treated with *C. bergamia* against *A. rolfsii* showing significant root destruction; (**j**) healthy aerial parts treated with EP5 against *F. oxysporum*; (**k**) unhealthy aerial parts treated with *P. granatum* against *S. sclerotiorum* showing stunted leaves; (**l**) severely unhealthy aerial parts treated with *C. bergamia* against *A. rolfsii* showing wilt and necrosis of leaves.

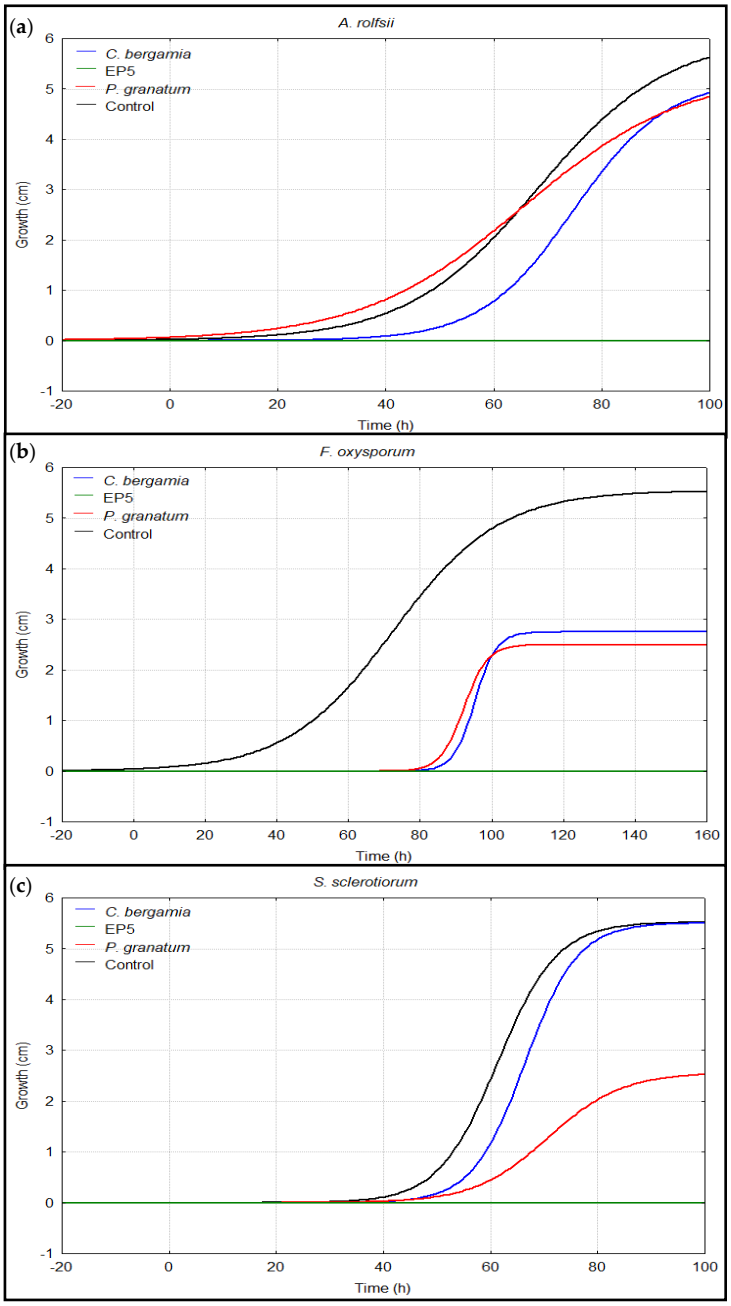


Figure 3. Growth kinetics of fungal pathogens on culture media amended with different putative inhibitory products (PIPs): (**a**) *Agroathelia rolfsii* growth; (**b**) *Fusarium oxysporum* growth; and (**c**) *Sclerotinia sclerotiorum* growth. Fungal growth on a culture medium not amended with PIPs was used as the control. The colored lines represent the fungal kinetics best fitted to the data based on Dantigny' model [41].

Moreover, the inhibitory effect of bergamot and pomegranate persisted until the end of the experiment, as the growth of fungal pathogens remained constant after 100 h. While the growth trends of *A. rolfsii* and *F. oxysporum* were similar in the presence of bergamot and pomegranate, *S. sclerotiorum*'s sensitivity was significantly different for these two PIPs. The τ values of bergamot (τ = 66.44 h), EP5 (τ = 480.00 h), and pomegranate (τ = 70.70 h) were higher than the control's value (61.34 h), suggesting that all the PIPs slowed the growth of fungal pathogens. While the τ values of bergamot and pomegranate were very similar, the final growth values of the fungal pathogens exposed to them were different. *S. sclorotiorum*'s final growth after exposure to bergamot was equal to that of the control, while

its final growth after exposure to pomegranate was lower. This suggests that bergamot lost its efficacy over time, while pomegranate did not (Figure 2).

3.2. Assessment of Effectiveness of PIPs Against Fungal Pathogens Under In Vivo Conditions

Figure 4 (as well as Tables S5–S7) presents the evaluation results of the efficacy of the PIPs as control methods against soilborne fungal pathogens that affect the roots of tomato plants grown in the greenhouse experiment. The graph in panel (a) describes the sensitivity or aggressiveness of the fungi used in the experiment when exposed to the PIPs. In particular, symptom manifestation on tomato roots, expressed as disease incidence (MkI), was overall better controlled when the PIPs were used against *F. oxysporum* (MkI = 11.46%) and *S. sclerotiorum* (MkI = 16.49), but it was less controlled against *A. rolfsii* (MkI = 32.76%) (Table S5). The graph in panel (b) highlights the overall performance of the different PIPs in controlling fungal pathogens, where EP5 and pomegranate were the most effective with MkI values, recorded from roots, of 4.51 and 9.20%, respectively. In contrast, bergamot was not effective because a high value of disease incidence was recorded (MkI = 25.00%) (Table S6).

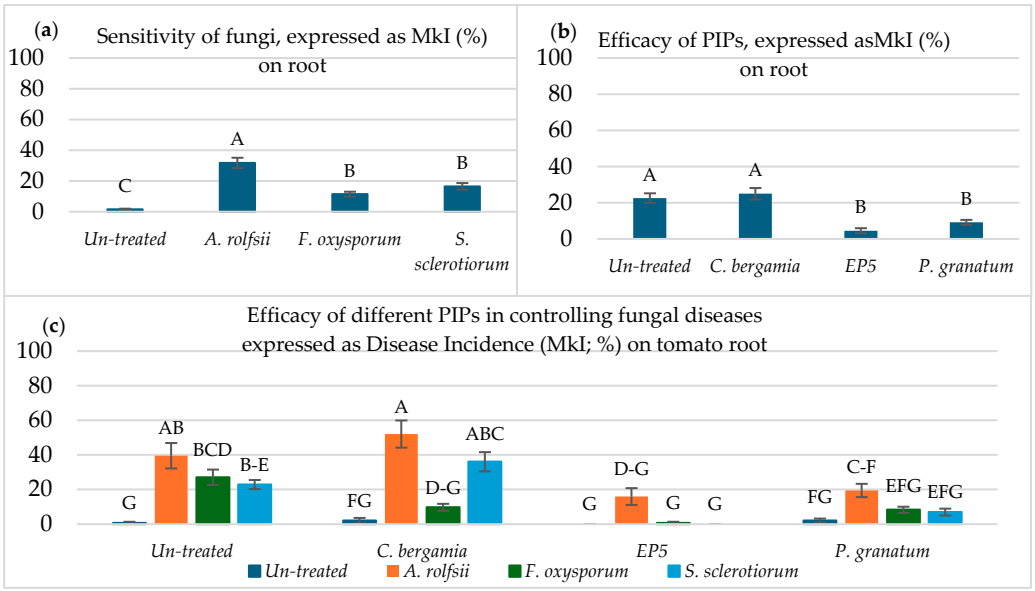


Figure 4. Efficacy of putative inhibitory product (PIP) treatments in controlling soilborne fungal pathogens in greenhouse-grown tomatoes, as determined by root phytosanitary status: (**a**) sensitivity of fungi exposed to PIPs, as assessed by disease incidence (MkI; %) on tomato roots; (**b**) effectiveness of PIPs in controlling fungal pathogens, expressed as disease incidence on tomato roots; and (**c**) interaction between PIP treatments and fungi, as assessed by disease incidence on tomato roots. Data were analyzed using one-way and factorial ANOVAs and Tukey's test ($p < 0.01$). Means sharing the same capital letter do not differ significantly.

Panel (c) displays the interaction between specific treatments and fungi, where EP5 was the most effective against all three fungal species, with disease incidence ranging from 0.0 (*S. sclerotiorum*; un-treated trial) to 15.97% (*A. rolfsii*). A similar trend could be observed from the trial with pomegranate, though its MkI values were slightly higher overall (ranging from 6.95 to 19.45% against *S. sclerotiourum* and *A. rolfsii*, respectively) than those obtained with the EP5 treatment. In contrast, among the tested PIPs, bergamot showed variable effectiveness against different fungi: it was effective in controlling disease incidence caused by *F. oxysporum* (MkI = 9.72%) in roots but and not effective against *A. rolfsii* (MkI = 52.08%) and *S. sclerotiorum* (MkI = 36.11%). Moreover, the disease incidence

values recorded when bergamot was used were higher than those recorded for plants not treated with any PIP but artificially inoculated with fungi (un-treated trial) (Table S7).

Figure S1 (as well as Tables S8–S10) reports the results specific to symptom manifestation on the collar of tomato plants, expressed as disease incidence (MkI; %). The results regarding the aggressiveness of the fungal pathogens (Table S8) in affecting the collar of tomato plants are similar to those presented in Figure 4 (Tables S5–S7). In particular, *A. rolfsii* was the most aggressive fungus, showing a mean disease incidence of 23.26%, while *Fusarium oxysporum* (MkI = 2.26%) and *S. sclerotiorum* (MkI = 1.39%) were the weaker fungal pathogens. Statistically, their damage levels were indistinguishable from the un-treated control (MkI = 0.00%) (Table S8). Table S9 displays the overall performance or efficacy of the PIPs across the three fungal pathogens. The bergamot treatment was statistically identical to the un-treated control, as the recorded disease incidences were 9.90 and 10.94%, respectively. In contrast, EP5 (MkI = 2.78%) and pomegranate (MkI = 3.30%) significantly reduced disease symptoms by roughly 70–75% compared with the averages obtained from the un-treated control and the bergamot treatment. Table S10 clearly reveals the successful and failed treatments, as determined by the specific interactions of the fungi and PIPs. In the presence of *A. rolfsii*, the un-treated tomato plants (MkI = 33.33%) and bergamot-treated plants (MkI = 36.11%) showed significantly high disease levels.

On the other hand, EP5 and pomegranate significantly reduced *A. rolfsii*-mediated disease incidence to 10.42 and 13.19%, respectively. Regarding *F. oxysporum* and *S. sclerotiorum*, disease incidence values remained significantly low (MkI ranged from 0.00 to 3.47%) across almost all treatments (EP5, pomegranate, and bergamot), making it difficult to detect which PIP treatment was more effective since the infection at baseline was negligible.

Figure 5, as well as Tables S11–S13, highlights the aerial phytosanitary status of tomato plants at the end of the growth cycle.

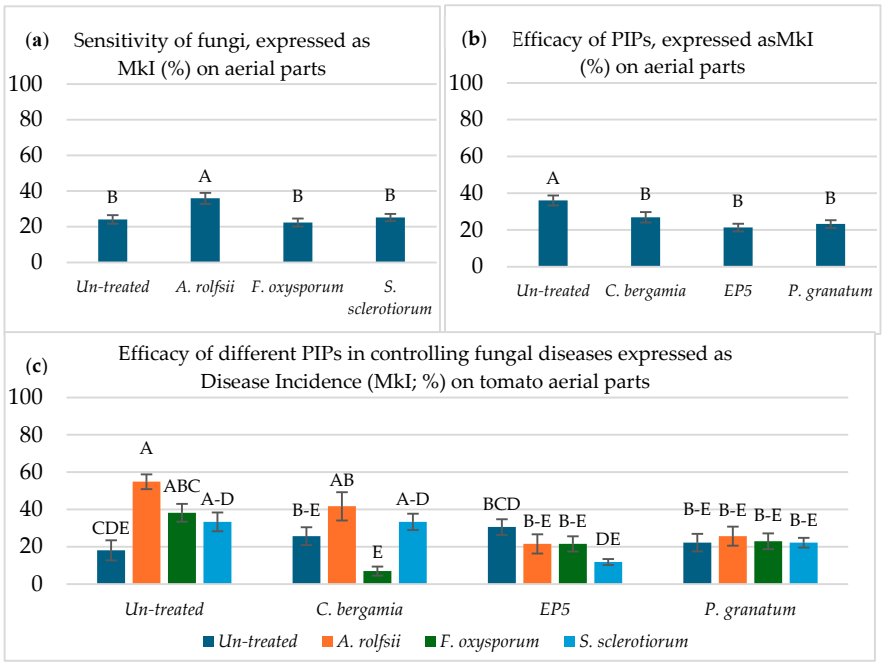


Figure 5. Assessment of the efficacy of putative inhibitory product (PIP) treatments in controlling soilborne fungal pathogens in greenhouse-grown tomatoes, as determined by the phytosanitary status of aerial parts: (**a**) sensitivity of fungi exposed to PIPs, as assessed by disease incidence (MkI; %) on tomato aerial parts; (**b**) effectiveness of PIPs in controlling fungal pathogens, expressed as disease incidence on tomato aerial parts; (**c**) interaction between PIP treatments and fungi, as assessed by disease incidence on tomato aerial parts. Data were analyzed using one-way and factorial ANOVAs and Tukey's test ($p < 0.01$). Means sharing the same capital letter do not differ significantly.

Graph (a) (Table S11) shows that disease incidence was significantly higher when *A. rolfsii* was used (MkI = 35.94%), while it was not significantly different from that of un-treated plants (MkI = 24.13%) when *F. oxysporum* (MkI = 22.40%) and *S. sclerotiorum* (MkI = 25.17%) were used. Graph (b) (Table S12) highlights that the PIPs did not produce phytotoxicity damage on the aerial plant part because their presence significantly protected tomato plants against fungal pathogens. In addition, the interaction between the PIPs and fungi shown in Graph (c) confirms the ability of the PIPs to protect the aerial plant part from damage caused by the presence of the fungi inoculated. In particular, the best PIP was EP5, with symptoms calculated as disease incidence ranging from 21.23 (*A. rolfsii*) to 11.81% (*S. sclerotiorum*). Also, pomegranate was effective, with disease incidence values ranging from 25.69 (*S. rolfsii*) to 22.22% (un-treated and *S. sclerotiorum*). In contrast, bergamot displayed selective protection activity; it was more effective against *F. oxysporum* (MkI = 6.94%) and less effective against *A. rolfsi* (MkI = 41.67%) and *S. sclerotiorum* (MkI = 33.33%) (Table S13).

Effects of PIPs and Fungal Pathogens on Plant Growth Length and Compactness

Figure 6 (as well as Tables S14–S19) presents the effects of PIPs and fungal pathogens on plant length, one of the growth indices evaluated in this experiment. The effects of fungal pathogens, PIPs, and their combinations did not follow a common trend. Specifically, the effects of fungal pathogens on final plant length revealed significant differences in the sensitivity of tomato plants to these pathogens. *F. oxysporum*, *S. sclorotiorum* and *A. rolfsii* reduced the growth of the plants compared with the control (82.79 cm), with *F. oxysporum*, *S.sclerotiorum*, and *A. roflsii* being slightly harmful (71.08 cm), moderately harmful (60.31 cm), and very harmful (49.67 cm), respectively, as observed in Graph (a) (Table S14). The effects of the PIPs were very variable, but there was a common trend toward growth promotion compared with un-treated plants (56.01 cm). Graph (b) (Table S15) shows that EP5 significantly promoted plant length, with a value of 77.32 cm, while growth promotion by pomegranate and bergamot was less significant, with values of 66.38 cm and 64.15 cm, respectively. Graph (c) (Table S16), in general, highlights the negative effects of fungal pathogens and the positive effects of PIP treatments. The presence of fungal pathogens, without any PIP treatment, significantly reduced plant growth, with values ranging from 37.83 (*A. rolfsii*) to 55.71 cm (*F. oxysporum*). The PIP treatments normalized the growth of the plants, thus limiting the negative effects of the presence of fungal pathogens. EP5 was the most effective PIP, with values ranging from 60.42 to 84.29 cm, while pomegranate and bergamot showed less pronounced effects, with values ranging from 52.19 to 81.33 cm and from 48.35 to 82.88 cm, respectively. Although the results depend on the different interactions between fungi and PIPs (indicating variability in fungus–PIP interactions), the un-treated plants and the plants treated with bergamot, pomegranate, and EP5 showed a similar negative trend with regard to *A. rolfsii*, with values of 37.83, 48.25, 59.19, and 60.42 cm, respectively. The results regarding tomato plant compactness followed a trend similar to that observed for final length. Graph (d) (Table S17) shows that *A. rolfsii* significantly increased compactness, suggesting a small growth of the plant (9.02 g cm^{-1}). *S. sclortiorum* and *F. oxysporum* showed a similar effect on compactness, but this effect was less significant when compared to the control, with values of 6.61, 5.37, and 4.11 g cm^{-1}, respectively.

Regarding the PIPs, plants treated with bergamot and un-treated plants showed a highest compactness, with values of 8.51 and 7.63 g cm^{-1}, respectively. In contrast, Graph (e) (Table S18) shows that EP5 and pomegranate did not increase compactness, and their effects were not significantly different, with values of 4.70 and 4.27 g cm^{-1}, respectively. Graph (f) (Table S19) clearly highlights the differences among the PIPs in relation to the fungal pathogens. While compactness in plants treated with EP5 and pomegranate remained

relatively stable, with values ranging from 3.95 (EP5 vs. *S. sclerotiorum*) to 5.3 g cm^{-1} (EP5 vs. *A. rolfsii*) for EP5 and from 3.74 (pomegranate vs. *S. sclerotiorum*) to 5.46 g cm^{-1} (pomegranate vs. *A. rolfsii*) for pomegranate, the plants treated with bergamot showed a wide range of compactness depending on the fungal pathogen inoculated, ranging from 3.69 (bergamot without any fungal inoculation) to 15.08 g cm^{-1} (bergamot against *A. rolfsii*). In general, the EP5 and pomegranate treatments reduced compactness to values almost equal to that of the un-treated and un-inoculated plants (4.14 g cm^{-1}). Moreover, the two PIPs reduced the compactness values observed in the un-treated and inoculated plants, which ranged from 7.78 (*S. sclerotiorum*) to 10.24 g cm^{-1} (*A. rolfsii*). Bergamot was the only PIP showing inconsistent effects, as it reduced the compactness of the plants treated with *F. oxysporum* (4.29 g cm^{-1}) and increased it in the plants treated with *S. scleortiorum* (10.97 g cm^{-1}) and *A. rolfsii* (15.08 g cm^{-1}).

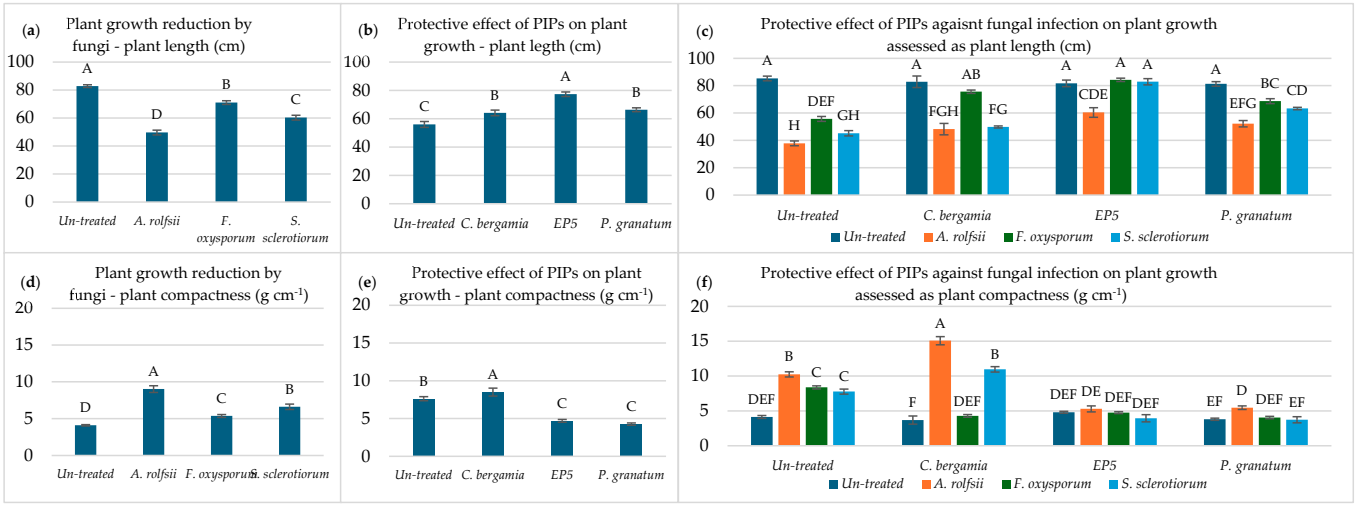


Figure 6. Effect of putative inhibitory product (PIP) treatments on tomato plant growth indices, as determined by plant length (cm) and compactness (g cm^{-1}): (**a**) reduction in plant growth caused by fungal pathogens, expressed in terms of plant length; (**b**) PIP protection on plant growth, expressed in terms of plant length; (**c**) effect of the interaction between fungal pathogens and PIPs on plant growth, as assessed by plant length; (**d**) reduction in plant growth caused by fungal pathogens, expressed in terms of compactness; (**e**) protective effect of PIPs on plant growth, expressed in terms of compactness; (**f**) effect of the interaction between fungal pathogens and PIPs on plant growth, as assessed by plant compactness. One-way and factorial ANOVAs (interaction between fungi and PIPs) and Tukey's test ($p < 0.01$) were performed; the means with the same capital letters indicate no significant differences.

3.3. Survival of Inoculated Fungi Assessed by Re-Isolation Frequency

Figure 7 (along with Table S20) reports the re-isolation frequency percentage (RIF; %) of artificially inoculated fungi, which better explains the MkI observed on roots. In particular, the graph highlights the high efficacy of EP5 (from 4.17 to 10.42%).

The same panel shows that between the other two PIPs, pomegranate was more effective in inhibiting fungal growth (from 10.42 to 31.25%) than bergamot (from 31.25 to 60.42%). Additionally, the R-IF values of artificially inoculated fungi were lower in the trials with EP5 and pomegranate than that of the un-treated trial, ranging from 41.67 (in the presence of *F. oxysporum* and *S. sclerotiorum*) to 85.42% (in the presence of *A. rolfsii*). Although all the PIPs showed general efficacy, the bergamot vs. *S. scelrotiorum* trial (45.83%) was the only trial with an RIF value higher than that of the un-treated trial (41.67%).

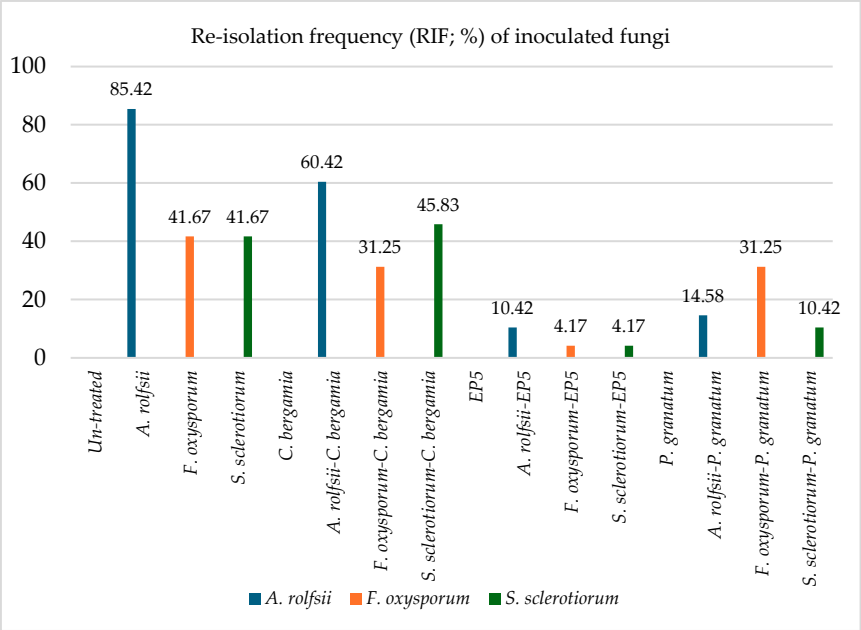


Figure 7. Percentage of inoculated fungal pathogens re-isolated from tomato roots.

4. Discussion

The experiment carried out in the present work highlights the effectiveness of potential plant products as sustainable control measures against several severe fungal pathogens under in vitro and in vivo conditions. Recent studies [29,30,32] have reported the potential use of natural fungicides that are environmentally friendly and safe for pollinating insects, livestock, and humans as alternatives suitable for organic and chemical-free agriculture, which is in agreement with the results obtained in greenhouse experiments on tomato seedlings. According to other studies, essential oils from thyme, oregano, and marjoram plants have shown strong antifungal activity against *Penicillium olsonii* [46,47]. Moreover, essential oils from oregano were reported to completely inhibit *Botrytis cinerea* growth under both in vitro and in vivo conditions, thus protecting tomato plants from disease-induced damage. More recently, Castello et al. [48] discussed results obtained from a greenhouse experiment where plant-based treatments were used to manage damping-off and root rot diseases caused by *Rhizoctonia solani* and *A. rolfsii*, respectively, on tomato plants. Therefore, the results of many investigations are consistent with those described in this work, which stated that, in the in vitro experiment, the most vulnerable fungal pathogen to PIP activity was *F. oxysporum*, characterized by the highest average sensitivity, while the most resistant pathogen was *A. rolfsii*. On the other hand, among the PIPs used, EP5, as a mineral product, was the most effective agent in controlling the fungi tested. Bergamot displayed specificity because it was quite effective against *F. oxysporum* but completely ineffective against *S. sclerotiorum*. Moreover, as similar results were obtained from the greenhouse experiment, it allowed us to infer that EP5 and pomegranate were the most effective natural products for inhibiting fungal growth and protecting roots and collars in live tomato plants, resulting in the lowest disease incidence values. In contrast, bergamot performed poorly under greenhouse conditions because its treatment resulted in higher disease incidence than untreated plants, suggesting that it may harm the plants or aid the fungus. Additionally, the antifungal activity of pomegranate and bergamot products differed due to the metabolites present in their peels. Many studies report the chemical composition and metabolic profiles of pomegranate and bergamot and demonstrate their high antifungal and antioxidant values [49,50]. Accordingly, our results on EP5, which contains minerals and silicates, encountered strong support from recent studies reporting the powerful effects of mineral

products such as zeolite and bentonite against root diseases caused by soilborne pathogens in tomato crops. These results indicated that zeolite and bentonite act as slow-release reservoirs for both water and defense-boosting ions by releasing silicon ions into the soil solution. Despite silicon not being an essential nutrient, it is absorbed by tomato roots and deposited beneath the cuticle and in cell walls, making root cells more resistant to fungal infections [51,52]. Therefore, EP5 is the best candidate for controlling soilborne fungi that affect plant roots, demonstrating consistent, broad-spectrum efficacy in both lab and greenhouse experiments. Pomegranate, as a plant PIP, is a viable alternative, while bergamot carries significant risk, as despite its effectiveness against specific fungi in the lab, it failed to protect roots and collars in live tomato plants and potentially worsened infections for certain pathogens. The efficacy of pomegranate against soilborne fungal pathogens of tomato plants has been positively reported by Leontopoulos et al. [53], who attributed its activity against *R. solani* and *F. oxysporum* f.sp. *lycopersici* to the high tannin content in its peel. Bergamot's reduced effectiveness against the same fungi can be linked to its high content of residual essential oils (volatile compounds) and flavonoids in peel waste [54,55]. Moreover, the phytosanitary status of the aerial parts of tomato plants at the end of their growth cycle allowed us to assess the PIP's potential to control soilborne fungal pathogens. The results are in line with those collected from roots and collars, confirming that *A. rolfsii* has been the most damaging pathogen, causing significantly higher disease incidence on root and collars, as well as on the aerial plant part, compared to the other fungi inoculated. Indeed, *A. rolfsii*, which affects the plant roots, also induces damage on the collar region, stem, and aerial parts, as confirmed by Aljabali et al. [56]. Moreover, the aerial symptoms caused by *F. oxysporum* and *S. sclerotiorum* were not significantly different from those observed in the un-treated control plants, suggesting that these pathogens primarily attack roots or cause low aerial stress. This study highlights that PIPs are phytotoxic to tomato plants and provide significant protection against fungal attacks. In particular, EP5 was the best PIP and most effective protectant across all agents evaluated because it was potent against fungi but gentle on plant tissue. This feature of EP5 makes it a highly valuable alternative for agricultural application. Otherwise, pomegranate can be used as an effective PIP as it provides good protection, and bergamot can be used selectively because of its high efficacy against *F. oxysporum* but inconsistent and poor performance against *S. sclerotiorum* and *A. rolfsii*. The results regarding the phytosanitary status of the tomato plants reflected the results on their growth, which was expressed as plant length (cm) and compactness (g cm^{-1}). The two parameters are not directly related, as it has been discovered that stressed plants tend to promote more biomass accumulation compared to plants undergoing elongation [57]. EP5 and pomegranate had stronger efficacy against the fungal pathogens and resulted in higher and less compact plants, while plants treated with bergamot were more sensitive to the fungal pathogens, displaying more compact and shorter profiles. The influence of zeolite and pomegranate on plant growth has been reported in other investigations. Zeolite significantly increases the height of the flowering stem, the diameter of the flower, the leaf area, and the chlorophyll content of tuberose plants [58], while pomegranate peel is able to control Fusarium wilt, leading to an improvement in the growth variables of tomato plants [59]. Moreover, the data shows that the aggressiveness of *A. rolfsii* and *S. sclerotiorum* leads to negative growth indices. The damage caused by these pathogens not only affect the growth of tomato plants [60] but also impact a wide range of botanical species, like *Mentha arvensis* [61]. Through the re-isolation frequency (RIF), this study determined the "colonization rate" of the pathogens within the roots of tomato plants artificially inoculated with fungal pathogens at the end of their growth cycles. The RIF data collected were found to correlate strongly with the MkI data collected from tomato roots. The low RIFs indicate reduced symptom presence,

confirming that the PIP treatments were able to prevent fungi from surviving or establishing themselves deep within roots. In particular, EP5 demonstrated high efficacy with very low fungal recovery. Pomegranate was also effective, significantly reducing fungal presence compared to controls; however, according to El Khetabi et al. [62] and Orak et al. [63], it allowed a few fungal species to grow. In contrast, this work reports that bergamot displays characteristics beyond being the least effective treatment; against *S. sclerotiorum*, it actually resulted in higher fungal presence than the controls, reinforcing the finding from previous research indicating that bergamot may promote certain fungal infections, as reported by Cebi et al. [50] and Whiley et al. [64].

5. Conclusions

The results discussed in this study are presented to evaluate plant-derived products as eco-friendly alternatives and mineral-derived products as suitable resistance inducers that elicit natural defenses inside tomato plants. Among the putative inhibitory products, EP5, which contains bentonite, emerged as the most effective treatment, likely due to the release of silicon to strengthen root cell walls. Pomegranate peel, due to its tannin content, also showed high efficacy, as demonstrated by its ability to effectively reduce the colonization of the fungal pathogens used in both experiments (in vitro and in vivo). In contrast, the effect of bergamot proved to be inconsistent and controversial because it targeted *F. oxysporum* in vitro but failed in vivo and could potentially worsen the infection. *Agroatelia rolfsii* was identified as the most aggressive pathogen, causing severe damage across all plant parts and making it difficult to limit its aggressiveness in both in vitro and in vivo experiments. Moreover, the successful treatments (EP5 and pomegranate) not only lowered disease incidence but also promoted better plant growth, thereby resulting in taller, healthier tomato plants, as demonstrated by their agronomic features. Overall, although EP5 and pomegranate represent safe, non-phytotoxic alternatives for sustainable, chemical-free agriculture, further experiments on the mechanisms governing host–pathogen interactions under natural conditions deserve attention. To conclude, scientific research in this area is required to provide policymakers with effective tools to guide farmers toward sustainable agriculture that is safe for consumers and the environment while remaining respectful of biodiversity.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture16080861/s1, Table S1: Sensitivity of Fungal species subjected to inhibiting activity of putative inhibiting products (PIPs) on artificial medium in in vitro conditions; Table S2: Inhibiting activity (IA) of putative in-hibiting products (PIPs) on artificial medium in in vitro conditions; Table S3: Inhibiting activity of putative inhibiting products (PIPs) on artificial medium against fungal pathogens in in vitro con-ditions; Table S4: Fungal growth (τ; time to attain a half of the maximum diameter expressed in hours) on the culture medium amended with putative inhibitory products (PIPs); Table S5: Disease Incidence determined based on root symptoms caused by inoculated fungi; Table S6: Disease In-cidence expressed as root symptoms based on efficacy of the putative inhibitory products (PIPs) to control inoculated fungi; Table S7: Effect of the interaction between the inoculated fungal pathogens and the putative inhibitory products (PIPs) in affecting root health; Figure S1: Efficacy of putative inhibitory products (PIPs) treatments in controlling soilborne fungal pathogens in greenhouse to-matoes, determined by the collar phytosanitary status: (a) Sensitivity of fungi exposed to PIPs, as-sessed by disease incidence (MkI; %) on tomato collar; (b) Effectiveness of PIPs in controlling fungal pathogens, expressed as disease incidence on tomato collar; (c) Interaction between PIP treatments and fungi, assessed by disease incidence on tomato collar. One-way and Factorial Anova, Tukey's Test ($p < 0.01$). Means sharing the same capital letter do not differ significantly; Table S8: Disease Incidence determined based on collar symptoms caused by inoculated fungi; Table S9: Disease In-cidence expressed as

collar symptoms based on efficacy of the putative inhibitory products (PIPs) to control inoculated fungi; Table S10: Effect of the interaction between the inoculated fungal path-ogens and the putative inhibitory products (PIPs) in affecting collar health; Table S11: Disease In-cidence determined based on aerial parts symptoms caused by inoculated fungi; Table S12: Disease Incidence expressed as aerial symptoms based on efficacy of the putative inhibitory products (PIPs) to control inoculated fungi; Table S13: Effect of the interaction between the inoculated fungal pathogens and the putative inhibitory products (PIPs) in affecting collar health; Table S14: Effect of the presence of the inoculated fungal pathogens on plant growth, assessed as plant length; Table S15: Protection promoted by the putative inhibitory products (PIPs) on plant growth, assessed as plant length; Table S16: Effect of the interaction between fungus and putative inhibitory products (PIPs) on plant growth, assessed as plant length; Table S17: Effect of the presence of the inoculated fungal pathogens on plant growth, assessed as plant compactness; Table S18: Protection promoted by the putative inhibitory products (PIPs) on plant growth, assessed as plant compactness; Table S19: Effect of the interaction between fungus and putative inhibitory products (PIPs) on plant growth, assessed as plant compactness; Table S20: Inoculated fungal pathogens re-isolated from the treated and un-treated root.

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Abbreviations

The following abbreviations are used in this manuscript:

PIP	Putative inhibiting product
MkI	McKinney index
RIF	Re-isolation frequency

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Objective 5. Evaluation of The *in vivo* efficacy of bergamot, pomegranate, and EP5 powders was also tested against common tomato weeds, specifically redroot pigweed (*Amaranthus retroflexus* L.), common lambsquarters (*Chenopodium album* L.), and black nightshade (*Article 5, ready for submission to* Weed research).

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Sustainable Weed Management in Processing Tomato: Evaluating the Bioherbicidal Potential of Agro-Industrial and Commercial Powders against Major Weeds and Their Impact on Early Crop Growth

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ABSTRACT

Background: The increasing demand for novel, sustainable, and eco-friendly weed management strategies has stimulated research into plant-derived products with bioherbicidal and allelopathic properties.

Experimental design: This study evaluated the allelopathic inhibitory potential of dried peel powders from bergamot (*Citrus* × *bergamia* (Risso) Risso & Poit.) and pomegranate (*Punica granatum* L.), along with the herbicidal potential of the EP5 commercial powder.

Methods: The powders were tested against three major tomato-associated weeds: *Amaranthus retroflexus* L., *Chenopodium album* L., and *Solanum nigrum* L. Furthermore, their potential phytotoxic effects on the growth and development of tomato (*Solanum lycopersicum* L.) seedlings were assessed to determine crop safety.

Results: The results demonstrated that bergamot peel powder exhibited the highest allelopathic suppression, reducing weed germination by 47.4% and seedling radicle length by 41.8% compared to the untreated control. *Amaranthus retroflexus* was found to be the most susceptible weed species, while *Solanum nigrum* showed moderate susceptibility. Regarding crop safetythe applied powders did not negatively affect tomato seedling height or biomass.

Conclusions: Overall, these findings highlight the potential of utilizing agro-industrial residues, particularly bergamot powders, and EP5 as promising components for integrated and sustainable weed management systems in tomato cultivation, promoting circular economy principles.

Keywords:

Allelopathy; Bioherbicides; Circular economy; Agro-industrial by-products; Sustainable agriculture; Weed control.

1. Introduction

Tomato (*Solanum lycopersicum* L.), belongiong to *Solanaceae* family, has become one of the most important and cultivated crops globally and across the Mediterranean region in the past decades. In 2019, approximatively 181 million tonnes of tomato have been produced from 5 Mha worldwide, with around 42 million tonnes originating from Mediterranean countries (Panno et al., 2021). Italy ranks as the world's second and European first producer of fresh tomato destined for processing. In 2021, Italy processed more than six millions of tonnes of fresh tomatoes, accounting for 16% of global and 52% of European production. The industrial turnover of this supply chain amounts to approximately 3.7 billion euros, 1.9 billion of which comes from exports. The larger quantity of fresh tomatoes harvested in Italy is attributable to both an increase in the cultivated surface area and an improvement in the average yield. In 2021, Northern and Central-Southern Italy represented the main sites in the country, producing 3.1 (51% of national production) and 2.9 (49% of national production) millions of tonnes, respectively (ISMEA, 2021). At the regional level, Apulia is the leading national producer of tomato. According to Confagricoltura Puglia (2026) the regional production represents the 40% of the national total, with the province of Foggia being the main area of interests. In fact, Foggia province production reaches approximatively 2.2 million tonnes, representing nearly the total of the regional production and making it the top-producing province nationwide, with a dedicated tomato cultivation area of 17,000 hectares (BASF Italia, 2026).

However, such intensive cultivation and high production density render this crop highly vulnerable to various threats. Globally, biotic, abiotic, and anthropogenic factors can cause severe yield and food losses, both in open field and post-harvest storage. Among these, weeds, alongside fungal and bacterial pathogens, and insects are the major factories causing food losses. Currently, it is estimated that weeds alone cause 34% of agricultural losses, while fungal pathogens and insect pests account for 20–40% and 38% of losses, respectively; however, these percentages further escalate in developing countries due to a lack of effective control measures and limited awareness within the agricultural community (Junaid and Gökçe, 2024). These losses represent a major concern given the increasing food demand of global population, which is projected to reach 9.8 billion by 2050, and 11.2 billion by 2010. (UN DESA, 2017).

Tomato is one of the most vulnerable crops to pests throughout all growth stages, making its management challenging. Pests lead to significant yield losses and occasional complete crop failure (Ivezić et al., 2025). In particular, weeds can cause up to 75% loss of tomato yield, while fungal pathogens can lead to 50-80% tomato fruit losses, , if not properly managed (Falade and Alabi, 2025; Laude, 2023). This high risk of yield reduction is driven by a wide variety of flora. In the Mediterranean area, where tomato crop is mostly transplanted, the associated weed communities are

commonly very rich of species, tough they are dominated by a small group of common species. These include barnyard grass (*Echinochloa crus-galli* (L.) P.Beauv.), pigweed (*Amaranthus* spp. L.), lamb's-quarters (*Chenopodium album* L.), knotweed/smartweed (*Polygonum* spp. L.), purslane (*Portulaca oleracea* L.), black nightshade (*Solanum nigrum* L.). Conversely, in field-seeded crops and in Northern countries, early-emergencing weeds, like black grass (*Alopecurus myosuroides* Huds.), wild oat (*Avena* spp. L.), ryegrass (*Lolium* spp. L.), and several species of the *Cruciferae* family (Juss.) and *Compositae* family (Giseke), are more frequent. Black nightshade (*Solanum nigrum* L.) alongside hairy nightshade (*S. sarrachoides* Sendtn.) and hoe-nightshade (*S. physalifolium* Rusby) in Spain, and silverleaf nightshade (*S. elaeagnifolium* Cav.) in Israel, is the key weed in all the countries. Among parasitic plants, broomrape (*Orobanche* spp.L.) poses severe problems in Spain, Portugal, Morocco, Italy and Israel, while field dodder (*Cuscuta campestris* Yunck.) is prevalent in Spain and Israel (Tei et al., 2003). In the Apulia region, weeds cause drastic yield reduction, ranging from 5 to 100% (Lops et al., 2021). The main weeds include parasitic plants like broomrape, broadleaf weeds like black nightshade, and pigweed, and grasses like barnyardgrass and bristlegrass (*Seteria* spp P.Beauv. L.) (ADAMA Italia, 2021).

In the past, the management of weeds, as well as fungal pathogens and insects, has mainly relied on the application of synthetic chemical pesticides to sustain crop yield (European Environment Agency, 2023). However, the intensive use of these pesticides is highly hazardous to the non-target organisms, the environment and human health (Akhter et al., 2023), leading to severe negative effects such as environmental pollution, soil degradation, water resources contamination, and biodiversity loss. Regarding human health, whether through occupational or food residue exposure, chemical pesticides are linked to chronic illnesses, including cancers, neuroglial diseases, and hormonal disruption (European Environment Agency, 2023). For instance, glyphosate, which is largely used in the Apulia region, is classified as "potentially cancerogenic" (Scortichini, 2020). Moreover, in 2024, the massive application of chemical pesticides leaded to severe honeybees mortality in the neighbour Molise region. Concerns regarding human health, environmental safety, ecosystems, and biodiversity have lead to new global and regional policies targeting chemical pesticide application (Romeh, 2018). Over the last decades, several Directives and Regulations have been implemented to reduce and standardize the use of chemicals in Europe, like Regulation (EC) No 396/2005 regarding Maximum Residue Levels (MRLs) in food products, or Regulation (EC) No 1107/2009 concerning the evaluation and commercialization of chemical pesticides. These regulatory frameworks have prompted the development of novel strategies to promote sustainable alternatives to traditional management, such as the Farm-to-Fork (F2F) Strategy, which is a core element of the European Green Deal (EGD).Beyond ensuring long-term food security for 530 million Europeans by 2050, the F2F Strategy

sets rigorous 2030 targets, including a significant 50%reductions in pesticide and fertilizer use, a decrease in food waste, and the expansion of organic farming and animal welfare standards (Hoiz and Esteves, 2026). Moreover, the EU economically supports this shift from conventional to eco-friendly agriculture through the CAP 2023-2027, with more than 2 billion euro are budgeted to support the maintenance of or shift toward sustainable agriculture (MASAF, 2026).

Consequently, minimizing food losses requires strategies that enhance food production while conserving natural resources (Junaid and Gökçe, 2024). Globally, Integrated Pest Management (IPM) and biological control are pivotal for managing crop biotic adversities. IPM combines biological, cultural, mechanical, physical, and chemical tools to minimize economic, health, and environmental risks. Chemical pesticides are applied as last resort when specific pest density thresholds are exceeded. Moreover, this management allows. Conversely, biological control totally bans the application of chemical pesticides, promoting natural mean, such as living organism and plant-derived substances (European Community, 2021; Romeh, 2018). Plant-derived products, like raw agricultural wastes (AWs), extracts and essential oils, represent a suitable alternative for managing biotic stresses (Akhter et al., 2023). For example, soil-applied orange (*Citrus sinensis* (L.) Osbeck) peel powdercontrols pre-emergent canarygrass (*Phalaris canariensis* L.) and cheeseweed mallow (*Malva parviflora* L.) (El-Wakeel and El-Metwally, 2020). Aqueous leaf extracts of false yellowhead (*Dittrichia viscosa* (L.) W.Greuter), river red gum (*Eucalyptus camaldulensis* Dehnh.), and white horehound (*Marrubium vulgare* L.) show high efficacy against animated oat (*Avena sterilis* L.), ear-fruited buckler-mustard (*Biscutella auriculata* L.), Maltese star-thistle (*Centaurea melitensis* L.), and short-spiked canary grass (*P. brachystachys* Link), (Mehdaoui et al., 2025). Essential oil of orange, Ceylon citronella (*Cymbopogon nardus* (L.) Rendle), and Java citronella (*Cymbopogon winterianus* Jowitt ex Bor) are reported to be effective against Indian goosegrass (*Eleusine indica* (L.) Gaertn.), smooth amaranth (*Amaranthus hybridus* L.), sourgrass (*Digitaria insularis* (L.) Fedde), and wild poinsettia (*Euphorbia heterophylla* L.) (Marzochi et al., 2025). In general, *Citrus* spp. powders, extracts, and essential oils, are highly applicable for the sustainable management of pests and weeds, with limonene acting as the primary active compound. High concentration of limonene are found in the peel of various *Citrus* spp., including bergamort (*Citrus* × *bergamia* (Risso) Risso & Poit.), lemon (*Citrus* × *limon* (L.) Osbeck), orange etc. (Kato-Noguchi and Kato, 2025). Similarly, pomegranate (*Punica granatum* L.) peel extract shows high potential as bioherbicide. According to Farag and Emam (2016) pomegranate peels crude juice significantly inhibit weed germination due to its high phenolic and flavonoid contents. This inhibition is primarily driven by allelopathy, a biological phenomenon where plant-derived secondary metabolites actively interfere with the growth and development of neighboring species.

Driven by the increasing interest in novel and sustainable plant-derived products for the management of weeds, we hypothesized that powders from bergamot and pomegranate AWs can serve as effective bioherbicides through their allelopathic potential. The recycling of these agro-industrial by-products minimizes environmental waste and aligns with circular economy principles by converting agricultural by-products into high-value ecological inputs. Additionally, to broaden the spectrum of eco-friendly alternatives, the commercial mineral powder EP5 (characterized by a 5% citric acid content) was included in this evaluation. Despite its original design as a fungal resistance inducer, its chemical profile suggests an interesting inhibitory activity against weeds. This hypothesis is supported by recent evidence in soft wheat, where citric acid applications successfully suppressed pre-emergent weed growth and seed germination without causing phytotoxic injuries to the crop (Alpi et al., 2025). Given these promising outcomes in cereal systems, testing EP5 in processing tomato serves to verify whether its herbicidal efficacy and crop safety can be successfully transferred to a high-value vegetable crop. Should these hypotheses be validated, it would demonstrate the potential of EP5 as a dual-purpose sustainable input. Therefore, a pot experiment was carried out to evaluate: 1) the allelopathic inhibitory potential of dried peel powders from bergamot and pomegranate, alongside the herbicidal potential of the EP5 commercial powder, against three major tomato-associated weeds, namely *Amaranthus retroflexus* L., *Chenopodium album* L., and *Solanum nigrum* L.; 2) their possible phytotoxic effects on early tomato seedling growth and development.

2. Materials and methods

2.1. Plant-derived and commercial powders preparation and target weed selection

Before starting the experiment, powders from agro-industrial wasres AWs, such as fruit peels of bergamot (*Citrus x bergamia* (Risso) Risso & Poit) and pomegranate (*Punica granatum* L.), were prepared at the at the Laboratory of Agronomy, University of Foggia (41°27′ N, 15°33′ E, 76 m a.s.l., Apulia region, Southern Italy). Specifically, pomegranate fruit peels were obtained as by-products of juice extraction from a farm located in the agricultural area of Foggia district (Apulia region, Southern Italy); bergamot peels were collected as essential-oil extraction by-products from a farm located in the agricultural area of Gioia Tauro (Calabria region, Southern Italy). Fruit peels were processed following the procedure described by Morea et al. (2025). First, they were thoroughly rinsed, then sliced into small pieces, and air-dried under room conditions until an approximate weight loss of 50%. The semi-dehydrated tissues were then transferred to a ventilated oven maintained at 30°C (FN 500 Sterilizer, Nuve, Ankara TR) until a constant dry weight was reached. Finally, the dried peels were ground using an electric mill (Geomil-50P) equipped with a 600 µm mesh screen at and stored in

opaque plastic containers under dark and dry conditions until use. In addition to the agro-industrial powders, the commercial powder EP5 (Phenom Biotech s.r.l., Milan Italy) was included in the experiment. Primarily marketed as a sustainable plant defence inducer against fungal pathogens, the chemical composition of EP5 consists of 30% bentonite silicates combined with a mineral fraction, including 10% iron, 6% zinc and 6.5% manganese sulphate (6.5%), and an organic acid matrix, including 5% citric acid.

Three key weed species were selected as targets for the experiment: *Amaranthus retroflexus* L., *Chenopodium album* L., and *Solanum nigrum* L. These specific targets were chosen due to the pressure they exert on the processing tomato crop, actively jeopardizing yields across the Foggia district. Moreover, they represent a critical management challenge due to their rapid adaptation and expanding resistance toward traditional synthetic chemical herbicides. To guarantee experimental uniformity and high germination quality, the seeds were sourced from specialized commercial seed suppliers.

2.2. Experimental layout and setup

A greenhouse pot-experiment was carried out at the University of Foggia (41°27′ N - 15°33′ E, 76 m a.s.l.), Apulia region, Southern Italy, under natural conditions of light and day/night temperature, from June to August 2024. Processing tomato (*Solanum lycopersicum* L.) seedlings, cv. “Taylor F1” (Nunhems Italy, Sant’Agata Bolognese, Bologna, Italy) were used. .

Tomato seedlings were transplanted in 1.5-L cylindrical plastic pots filled with a commercial substrate, under either weedy (*A. retroflexus*, *C. album*, and *S. nigrum*) or weed-free conditions, and under either treated (bergamot, pomegranate and EP5 powders) or un-treated soil conditions.

For the experiment, pots were prepared by placing two layers of non-woven fabric at the bottom to prevent soil loss and filling them to approximately 2/3 of their height (6 cm) with “Universal Gaia” substrate (Agrochimica S.p.A., Bolzano, Italy). 2.5 g of each plant powder were placed on the soil surface of the weedy and powder-treated pots, respectively, and covered with 1.0 cm of soil layer. Each pot was then watered and covered with a plastic film. After 72 hours, one tomato seedling was placed at the centre of the pot in both the weedy and weed-free pots, as well as in both the powder treated and un-treated pots and covered with an additional 1.0 cm soil. 30 Weed seeds were sown equidistantly at the same deep of tomato seedling. Weed-free and un-treated pots served as controls. The experiment was arranged according to a completely randomized factorial design (CRD) with three weed species (*A. retroflexus*, W_{Ama}; *C. album*, W_{Che}; *Solanum nigrum*, $W_{Sol,}$ no weed or weed-

free control, W_0) x four powder types (bergamot, P_B; pomegranate, P_P; EP5, P_{EP5}; no powder or un-treated control, P_0) x twenty-four replicates, for a total of 384 experimental units. The combination of the two experimental factors, weed (W) and powder (P), resulted in the twelve experimental treatments reported in Table S1.

The number of germinations for both wheat kernels and weed seeds was recorded every 48 hours over a 45-day period.

To evaluate the impact of powders on seed germination (Božena, 2023), a set of germination-related parameters was determined only regarding weeds, as tomato was transplanted and not sown. These parameters include the final germination percentage (FGP, %), the germination index (GI) and the mean germination time (MGT, days). Specifically, the final germination percentage (FGP) was calculated according to the following formula:

$$FGP = \frac{\Sigma n}{TN} x\ 100 \quad [1]$$

where Σn is the total number of germinated seeds; TN is the total number of seeds left to germinate.

The mean germination time (MGT) was calculated according to Ellis and Roberts (1981) using the following formula:

$$\mathrm{MGT} = \frac{\sum(\mathrm{G_I\ x\ T_i})}{\mathrm{N}} \quad [2]$$

where G_i is the number of germinated seeds on day *i*, T_i is the day number from the start of the experiment, and N is the total number of seeds germinated at the end of the experiment.

The germination index (GI) was calculated according to Maguire (1962) using the following formula:

$$\mathrm{GI} = \sum \frac{\mathrm{G_i}}{\mathrm{T_i}} \quad [3]$$

where G_i is the number of germinated seeds on day i, and T_i is the day number from the start of the experiment until the day of the specific count.

To evaluate the impact of powders on weed and tomato seedling growth (Božena, 2023), a set of growth-related parameters was determined, including the root (RDM, % fw), shoot (SDM, % fw), and total dry matter content (TDM, % fw), the seed vigor index (SVI), the plant inhibition index (PII, %) and the biomass accumulation inhibition (BAI, %). Due to their dimension, for weeds only TDM was taken. The dry matter content for each seedling component was calculated according to the following formula:

$$\mathrm{DM_i(\%\ fw)} = \frac{\mathrm{DW_i\ (g)}}{\mathrm{FW_i\ (g)}} \quad [4]$$

where DW is the dry weight, FW is the fresh weight and i refers to the root (R), shoot (S) or total (T).

The seed vigor index (SVI) was calculated according to Al-Jibouri et al. (1958) using the following formula:

$$SVI = FGP\ (\%)\ x\ Total\ seedling\ length\ (cm) \quad [5]$$

The plant length inhibition (PLI) was calculated according to Ali et al. (2015) using the following formula:

$$PLI = \frac{Control\ seedling\ length\ (cm) - Treated\ seedling\ length\ (cm)}{Control\ seedling\ length\ (cm)}\ x\ 100 \quad [6]$$

The biomass accumulation inhibition (BAI) was calculated according to Ismil et al. (2019) using the following formula:

$$BAI = \frac{Control\ seedling\ fresh\ weight\ (g) - Treated\ seedling\ fresh\ weight\ (g)}{Control\ seedling\ fresh\ weight\ (g)}\ x\ 100 \quad [7]$$

2.3. Statistical analysis

All experimental data were first checked for normality and homogeneity of variances using the Shapiro–Wilk ($p \leq 0.05$) and Bartlett ($p \leq 0.05$) tests, respectively, and then tested for differences using analysis of variance (ANOVA). The statistical significance of the differences between the means was analyzed using Tukey's honest significant difference (HSD) post hoc test at the 5% probability level. Data from the first *in vivo* experiments were analyzed by two-way ANOVA to examine the effects of Weed (W: four levels, W_0, W_{Ama}, $W_{Che,}$ W_{Sol}), Powder (P: four levels, P_0, P_B, P_P, P_{EP5}), as well as their interactions (W x P). Particularly, for the *in vivo* experiments, the datasets related to wheat and the datasets related to weeds were analyzed independently. This distinct approach was implemented to separately evaluate the potential phytotoxic effects of the powders on the germination and early development of wheat and their actual allelopathic and bioherbicidal efficacy against the target weed species. All statistical analyses were performed using the JMP software package, version 16.2 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Weeds

3.1.1. Germination parameters

For all the germination-related parameters, the experimental factors Weed (W), Powder (P), and their interaction (P x W) resulted always significant. Among the selected weeds, *A. retroflexus* (W_{Ama}) showed the highest final germination percentage (FGP), resulting in being significantly less susceptible to the treatments with the powder (FGP = 70.8%) compared to *S. nigrum* (W_{Sol}) (FGP = 26.6%) and *C. album* (W_{Che}) (FGP = 25.5%). All the powders reduced significantly the germination of the weeds, halving their FGP. In particular, bergamot (P_B) was the powder with the highest allelopathic effect, reducing the FGP to 29.7%, compared to the untreated weeds (62.6%). Although

significant, pomegranate (P_P) and EP5 (P_{EP5}) showed less allelopathic effects, reducing the FGP to 35.2 and 36.3%, respectively. Interestingly, across the entire thesis, W_{Ama} treated with P_{EP5} was the only one with a FGP higher (FGP = 80.0%) than the respective control (72.6%). W_{Ama} was the only weed with a low FGP reduction due to the treatments (ranging from 59.9, when treated with P_B, to 80.0%, when treated with P_{EP5}), while W_{Che} (ranging from 10.8, when treated with P_B, to 13.2%, when treated with P_{EP5}) and W_{Sol} (ranging from 15.8, when treated with P_{EP5}, to 22.8%, when treated with P_P) reduction higher. Thus, W_{Sol} was the only weed susceptible to P_{EP5} (Table 1).

Regarding MGT, Weeds exhibited significantly different responses to one another. W_{Ama} Also in this case, resulted to be the less susceptible to the allelopathic effects of the P, followed by W_{Che} and W_{Sol}, as the number of days taken for reaching half of their final germination were 8.1, 12.4, and 16.6 days, respectively. No strong significant differences were observed among the treated and untreated thesis. Moreover, P_P and P_B reduced the MGT to 12.0 and 12.2 days, respectively, compared to P_0 (12.6 days), meaning that the seeds treated with these powders took less days to reach half of their final germination. Instead, P_{EP5} was the only P to slightly increase MGT to 12.7 days. The treatments showed no significant effect on W_{Ama} (MGT ranging from 7.9, when untreated, to 8.4 days, when treated with P_{EP5}) and W_{Sol} (MGT ranging from 16.3, when treated with P_B, to 17.2 days, when treated with P_{EP5}). Instead, P_P and P_B significantly reduced MGT, while P_{EP5} slightly reduced MGT, to 11.8, 12.0, and 12.4 days, compared to the control (MGT = 13.5days) (Table 1).

Among the weeds, W_{Ama} was statistically faster than the other weeds regarding germination, since its germination index (GI) was equal to 2.7. Instead, W_{Che} and W_{Sol} had a similar response to the treatments, since their observed GI was 0.6 and 0.5, respectively. Untreated weeds germination was significantly faster than treated weeds (GI = 1.8). P_P (GI = 1.2) and P_{EP5} (GI = 1.2) slowing effects were statistically the same, while P_B (GI = 1.0) resulted to be more allelopathic than the other two powder in terms of GI. While W_{Che} and W_{Sol} were affected by all the powder compared to P_0, W_{Ama} was susceptible only to P_B. In particular, W_{Ama} GI values ranged from 2.3, when treated with P_B, to 3.0, when treated with P_{EP5}; W_{Che} GI values ranged from 0.3, when treated with the tree powders, to 1.5 in the control; W_{Sol} GI values ranged from 0.4, when treated with P_{EP5}, to 0.9 in the control (Table 1).

W_{Ama} was more vigour than W_{Che} and W_{Sol}, as the observed SVI were 848.9, 277.8, and 307.2, respectively. No differences among the powders were observed in terms of SVI, as P_B (SVI = 330.9), P_P (SVI = 325.0), and P_{EP5} (SVI = 377.6) reduced statistically similarly the vigour of the weeds compared to the control (SVI = 878.4). Like for the other parameters, the powders allelopathic effect changed based on the weed. W_{Ama} was more sensitive to P_P (SVI = 693.5), W_{Che} to P_B (SVI = 86.3), W_{Sol} and P_{EP5} (SVI = 139.0) (Table 1).

3.1.2. Growth parameters

Among the growth-related parameters, the experimental factors Weed (W), Powder (P), and their interaction (W x P) were significant on root, shoot, and total length (RL, SL, and TL), but not on the total dry matter (TDM) of the weeds.

In general, *S. nigrum* (W_{Sol}) roots grew significantly more than *A. retroflexus* (W_{Ama}) and *C. album* (W_{Che}), as their RL was 4.4, 2.8, and 3.2 cm, respectively. No significant differences were observed among the treatments with bergamot (P_B) (RL = 2.3 cm), pomegranate (P_P) (RL = 2.6 cm), and EP5 (P_{EP5}) (RL = 2.7 cm), although they significantly reduce the RL compared to the control (P_0) (RL = 5.5 cm). The data regarding the interaction W x P showed that W_{Ama} roots were more sensitive to P_B (RL = 2.1 cm) and P_{EP5} (RL = 2.5 cm), and less to P_P (RL = 2.6 cm), compared to the control (RL = 4.1 cm). Instead, W_{Che} roots showed the same sensibility to P_B, P_P, and P_{EP5}, with a RL equal to 2.2, 2.5, and 2.5 cm, respectively, compared to the control (RL = 5.0 cm). W_{Che} roots showed a different sensibility to the powders compared to the other weeds, as the RL of the plants treated with P_B and P_P were to 2.8 and 2.6 cm, respectively, while the RL of the plants treated with P_{EP5} was 3.3 cm, so the reduction compared to the control (RL = 8.9 cm) was higher with P_B and P_P than P_{EP5} (Table 2).

W_{Ama} shoot was the highest among the weeds, followed by W_{Che} and W_{Sol}, with a SL equal to 9.1, 7.2, and 5.5, respectively. Differently from RL, SL reduction statistically changed among the powders. In particular, P_B (SL = 8.4 cm) showed the least allelopathic effect, while P_P (SL = 7.0 cm) and P_{EP5} (SL = 7.9 cm) the highest, compared to P_0 (SL = 9.0 cm). In fact, P_B (SL =9.7 cm) showed no allelopathic effect on W_{Ama} compared to the control (SL = 10.1 cm), while P_P and P_{EP5} did, with a SL reduction to 8.1 and 8.8 cm, respectively. W_{Che} (SL= 8.5 cm) reduction of SL was statistically higher with P_{EP5} (SL= 6.1 cm) and P_P (SL= 6.7 cm) than with P_B (SL= 7.3 cm). Like W_{Che}, W_{Sol} showed a significant higher sensibility to P_P and P_{EP5} than P_B, which reduced SL to 4.9, 5.2, and 5.6 cm, respectively, compared to the control (SL = 6.9 cm) (Table 2).

In general, W_{Ama} was statistically the least sensitive to the treatment in terms in TL (TL = 11.8 cm), while W_{Che} (TL = 10.3 cm) and W_{Sol} (TL = 10.0 cm) response to the powder was similar. Although they were not statistically different, P_P resulted to be slightly more allelopathic than P_{EP5} and P_B, as they reduced the TL to 9.6, 10.5, and 10.7 cm, respectively, compared to the control (TL = 14.5 cm). W_{Ama} TL was reduced the most by P_P and P_{EP5}, and least by P_B, reaching values of 10.7, 11.3, and 11.8 cm, respectively, compared to the control (TL = 14.2 cm). Instead, P_{EP5} was more allelopathic than P_P and P_B on W_{Che}, as they reduced the TL to 8.6, 9.2, and 9.5 cm, respectively, compared to the control (TL = 13.5 cm). Like the other two weeds, W_{Sol} was most affected by P_P (TL = 7.5 cm), but,

differently from them, the powder with the second most allelopathic effect was P_B (TL = 8.4 cm), although not statistically different from the effect of P_{EP5} (TL = 8.5 cm). Anyway, all the three powders reduced the TL of W_{Sol},compared to the control (TL = 15.8 cm) (Table 2).

Figure 1 shows the results obtained regarding the total dry matter (TDM). TDM was not significantly different among the weeds, but it was influenced by the treatments (Figure 1a). P_{EP5} and P_B were the most allelopathic powder, significantly reducing the TDM to 16.1 and 18.3%, respectively, compared to P_P, which slightly significantly reduced the TDM to 19.9%. Without any powder, the TDM observed was 25.8% (Figure 1b). Across all experimental treatments, no significant differences were recorded (Figure 1c).

3.1.3. Growth indices

Both plant length inhibition (PLI) and biomass accumulation inhibition (BAI) resulted significantly affected by the experimental factor Weed (W), Powder (P), and their interaction (W x P).

Among the weeds, *S. nigrum* (W_{Sol}) and *C. album* (W_{Che}) resulted statistically more inhibited than *A. retroflexus* (W_{Ama}), as their PLI were 37.2, 29.44, and 15.5%, respectively. Pomegranate (P_P), bergamot (P_B), and EP5 (P_{EP5}) significantly inhibited the length of the plants, with a PLI equal to 38.0, 37.2, and 34.3%, respectively, although no significant differences were recorded among them (Table 3).

The BAI observed for the weed was significantly lower for W_{Ama} (BAI = -291.9 %) and W_{Sol} (BAI = -225.2%) than W_{Che} (BAI = -60.4%). Regarding the powder, P_B was the treatment that gave the significantly lower BAI, followed by P_{EP5} and P_B, whose BAI was statistically similar to each other but slightly different from P_B, with values equal to -325.5, -296.6, and -148.0%, respectively (Table 3).

3.2. Tomato

3.2.1. Growth parameters

Tomato growth-related parameter, specifically length and dry matter, showed different trends in relation to the experimental factors. Tomato root length (RL) wasn't affected by weed competition (W), powder inhibition (P), and their interaction (W x P). Instead, shoot and total length (SL, and TL, respectively) resulted to be affected by the W. *S. nigrum* (W_{Sol}) was the more competitive, followed by *A. retroflexus* (W_{Ama}) and *C. album* (W_{Che}). In particular, W_{Ama} reduced SL and TL by 11 and 10%, W_{Che} by 7 and 8%, and W_{Sol} by 14 and 13%, respectively. Instead, the experimental factor P didn't significantly affect the length of any part of the plant. The interaction W x P also showed no influence on RL, SL, and TL (Table 4).

While SL and TL showed susceptibility to W competition, neither W, P, and their interaction W x P, significantly affected the root, shoot, and total dry matter (RDM, SDM, and TDM, respectively) (Figure 2). Specifically, Figures 2a and 2b illustrate the effect of W and P on RDM, highlighting that tomato roots were not affected by the competition of none of the weeds and the allelopathic effects of the powders. As well as Figures 2a and 2b, Figures 2c and 2d, and Figures 2e and 2f, show that no competition of the weeds nor allelopathic effect of the powders were observed on SDM and TDM, respectively. In general, although not significantly, a slight change in growth-related parameters was observed under powder treatments. Regarding the length, pomegranate (P_P) slightly promoted the RL, while bergamot (P_B) and EP5 (P_{EP5}) reduced it, while all the powders reduced SL. TL showed the same response to the powders observed on RL. Regarding the dry matter, the powders always reduced it, except for P_B, which promoter RDM (Table 4) (Figure 2d).

3.2.2. Growth indices

As observed for the growth parameters, also plant length inhibition (PLI) and biomass accumulation inhibition (BAI) response to the experimental factors was different. Table 5 shows that W inhibited the growth of the plant compared to the control (W_0). *S. nigrum* (W_{Sol}) (PLI = 18.5%) and *A retroflexus* (W_{Ama}) (PLI = 17.3%) competition resulted in a significant inhibition when compared to the control (PLI = 7.8%), while *C. album* (W_{Che}) competition was less significant (PLI = 13.5%). While W experimental factor resulted to be significant, P factor and their interaction W x P wasn't significant. None of the powders inhibited the length of the plants were compared to the untreated control (P_0). Bergamot (P_B), pomegranate (P_P), and EP5 (P_{EP5}) observed inhibition on plant length were 17.0, 14.7, and 15.4%, respectively; while P_0 observed inhibition was 10.0%.

Instead, BAI resulted not to be influenced by any of the experimental factor nor their interaction. Although not significantly, W slightly inhibited biomass accumulation. As observed for PLI, W_{Sol} (BAI = 18.1 %) and W_{Ama} (BAI = 17.9%) competition resulted in a significant inhibition when compared to W_0 (BAI = 3.2%), while W_{Che} competition was less significant (BAI = 10.0%) (Figure 3a). Figure 3b illustrates the effect of the treatments with the P, highlighting that P_B, P_P, and P_{EP5} slightly inhibited the bioaccumulation in tomato plants. P_P resulted the most inhibiting powder, followed by P_B and P_{EP5}, causing an inhibition of 18.1, 14.1, and 10%, respectively, compared to P_0 (BAI = 7.0%). Interestingly, across all experimental treatments, tomato plants growth in weed-less condition and treated with P_B (BAI = -2.4%) and P_{EP5} (BAI = -5.6%) were the only ones with a negative BAI, meaning a promotion of bioaccumulation (Figure 3c).

Instead, BAI was not influenced by weed, powder, or their interaction. This enforces that the weeds weren't capable to affect any parameter of tomato plants due to their low competition and the effect of the powder on them.

4. Discussion

Food production and safety have always been a key point for the agriculture and policies. To ensure a level of production capable to satisfy the food demand of the global population, farmers have relied for decades, starting from the "Green Revolution", on the application of chemical pesticides for the management of fungal pathogens, weed, and insects (Pereira, 2026). The application of chemicals in the conventional management of these pests was forced by the need to guarantee an adequate yield by reducing the losses in open field and post-harvest (Nicolopoulou-Stamati et al., 2016). Although useful for agriculture, chemical pesticides application reduced year by year in the last decade due to their negative effects for human health, environment, and biodiversity. These pesticides can be potentially dangerous for human health as most of them are reported to cause chronic illnesses, hormonal alteration, and even neurological disorders, like autism or ADHD (Richardson et al., 2015). The level of dangerous changed based on the type of chemical pesticide an exposure. Spray-applied pesticides are the most dangerous, as they can spread be inhaled easily. Another dangerous way of exposure is represented by indirect ingestion. In fact, chemicals can accumulate into fruits and vegetables (Bansal, 2025). Regarding the environment and biodiversity, most of the time the traditional pesticides accumulate into the soil, altering the rhizosphere microbiome and affecting as the positive and useful microorganism. Moreover, the application can affect also nontarget organism, like bees and fish. The accumulation into surface and underground water represents a risk also for humans that can drink or eat animals that drank that water (Ahmad et al., 2024). In fact, chemical pesticide can accumulate into the flesh of the animals, ending through the food pyramid in consumer body (Nicolopoulou-Stamati et al., 2016; Zhou et al., 2025). Because of these negative effects, consumers and policies started to prefer and promote a novel and sustainable modern agriculture. Starting from the last century, the EU imposed increasingly strict laws, from establishing maximum residues level into the food, to banning chemical pesticides. Without the chemical tools, crop yield could not be enough to ensure the increasingly global food demand. In fact, according to FAO (2025), the global population will reach 10 billion of people by 2050. For this reason, modern agriculture is based on the application of sustainable means, represented mainly by plant-derived products and biocontrol agents (BCAs), capable to reduce the losses caused by fungal pathogens, weed, and insects. Among the plant-derived products, agricultural wastes (AWs) are now under study for their potential.

The application of AWs could be the perfect alternative to combine both environmental and economic sustainability (Koul et al., 2022). In facts, applying AWs, the farmers could re-use all the parts of the plants and fruit that had no commercial values, making them part of the circular economy. In facts, this alternative management could save trillions of dollars spent on the correct management of AWs. Moreover, this reuse of AWs can prevent their negative management. For instance, their negative processing is a global issue since its vast majority is currently burned or buried in soil, causing pollution of air, water and global warming (ICL Group, 2025; Koul et al., 2022). Among the AWs, *Citrus* spp. and pomegranate (*Punica granatum* L.) are object of many studies for their potential application against weed. For instance, Suwanagul et al. (2016) proved that the application of orange (*Citrus reticulata* Blanco) peel crude extract reduced the growth of the weed.

Based on this information, we tested bergamot (*Citrus* × *bergamia* (Risso) Risso & Poit.), pomegranate, and the commercial sustainable product EP5, composed primarily of bentonite, against the weeds of tomato *in vivo* condition by carrying out an experiment in greenhouse. The experiment aims to manage in a sustainable way the weeds of tomato as it's one of the main crops of Foggia province. In particular we tested both thire allelopathic effect on *Amaranthus retroflexus* L., *Chenopodium album* L., and *Solanum nigrum*, and their phytotoxicity on tomato plants. The evaluation was based on parameters related to germination, length, dry matter, and growth indices for weeds, while only on parameters related to length, dry matter, and growth indices for tomato, since tomato seedlings were used for the experiment, and not the seeds.

In general, each powder showed allelopathic effects on the germination parameters of the weeds, although the results changed based on the specific weed. In particular *Amaranthus retroflexus* was the least sensitive to the treatments, while *Chenopodium album*, and *Solanum nigrum* sensibility to bergamot, pomegranate, and EP5 was statistically the same, except for the mean germination time (MGT), since *S. nigrum* was more sensitive than *C. album*. Bergamot, pomegranate, and EP5 effects were the same for almost all the parameters. Bergamor resulted to be the most allelopathic powder regarding final germination percentage (FGP) and germination index (GI), while for the other parameters it shares the same effects with the other two powders. Interestingly, pomegranate was the only powder to promote a germination-related parameter, MGT, across all experimental treatments. Regarding FGP, EP5 had a promoting effect on *A. retroflexus*, the lowest inhibiting effect on *C. album*, and a strong inhibiting effect on *S. nigrum*, while bergamot was the most inhibiting powder on both *A. retroflexus* and *C. album*. The efficacy of bergamot can be related to its peel content in limonene. In fact, Ugolini et al. (2024) proved that the high content in limonene of orange peel is related to the inhibition of the germination of *C. album*. Regarding EP5, its promoting effect on *A.*

retroflexus can be related to its composition. In fact, zeolite could enhance seedling growth (Yılmaz et al., 2014).

Although MGT and GI are usually linked, the experiment showed that EP5 was the most allelopathic powder in terms of MGT, while bergamot in terms on GI. Taking into account that EP5 was less allelopathic than bergamot in terms of FGP, this may suggest that weeds treated with EP5 took more time than bergamot treated ones to reach half their final germination just because the final number of germinated weeds treated with EP5 is higher. The differences between the two parameters reflected in the different susceptibility of the single weed to the same powder. *A. retroflexus* was not affected by powders regarding MGT an GI, except by bergamot on GI. Instead, *C. album* sensibility to the powder was the same regarding the two parameters. *S. nigrum* was the only weed whose MGT was not affected by powder, but GI was affected by them. Also in these cases, the different trends between the two parameters are related to the differences in final germinated seeds.

Although bergamot affected FGP more than the other powder, no significant difference was observed among the plant treated with the powders. This may suggest that the lower effect of pomegranate and EP5 than bergamot on FGP was balanced by the total length (TL), as no significant differences were observed among the treatments with the powders.

In general, the weeds response to the treatments was different in terms of root, shoot, and total length (RL, SL, and TL), while the powders showed the same allelopathic effects, except for SL. Regarding the RL, *S. nigrum* was the more resistant to the treatments, while no differences in term of sensibility were observed between *A. retroflexus* and *C. album*. The three weed roots showed different sensibility to the powders, as *A. retroflexus* was affected the most by bergamot and EP5, *C. album* by all of them, and *S. nigrum* by bergamot. In facts, although statistically similar, bergamot was more allelopathic on the roots than the other two powders. Also in this case, the effect of bergamot on RL could be related to limonene content. According to Fagodia et al. (2017), limonene and citral contained in *Citrus* spp. peel significantly reduced weed RL. Moreover, the allelopathic resistance of *S. nigrum* have been already reported by Henriques et al. (2006). According to them, *S. nigrum* roots demonstrate remarkable resistance to environmental stress and allelopathic chemicals, primarily due to robust enzymatic defence mechanisms and their own diverse secondary metabolites

While *S. nigrum* roots were the least affected by the treatments, its shoot was the most affected by the powders, followed by *C. album* and *A. retroflexus*, suggesting that the allelopathic effect of the powder manifested primarily on the shoot for *S. nigrum*, and on the roots for *A. retroflexus* and *C. album*. The effects of the powder on the shoot were clearly different compared to the roots. For instance, bergamot resulted to be the most allelopathic powder on the RL and the least on the SL, while pomegranate was the second most allelopathic powder on RL and the first on SL, and EP5 the

least allelopathic powder on RL and the second on SL. In particular, pomegranate affected the most *C. album* and *S. nigrum*, while EP5 *A. retroflexus*.

While regarding the RL *S. nigrum* resulted to be the less sensitive to the allelopathic effects of the powders, the TL data revealed that *A. retroflexus* is, in general, the least sensitive, as the reduction of the RL is balanced by SL, and so *S. nigrum*, as it resulted to be the most sensitive. Similarly, the allelopathic effect of the powder on TL was the result of the combination of the allelopathy on RL and SL. Bergamo was the most allelopathic on the RL, but its least allelopathic effect on the SL made it the least allelopathic powders overall. Instead, pomegranate trend was opposite to bergamot, as it resulted to be the powder with the most allelopathic effect on SL and TL. This also suggest that, in general, the SL is the preponderant component of the TL, as the allelopathic effects observed on the SL were also reflected in the TL.

Regarding the TDM, the weeds were all affected the same way by the powders, as no significant differences were observed. Instead, regarding the powders, EP5 and bergamot significantly reduced the total content of dry matter, while pomegranate effect on TDM was less significant.

The data collected regarding the TL were confirmed by PLI regarding the weeds. A high PLI, being calculated based on the control of each weed, indicates how much the length of the plant is inhibited by the treatment. Compared to the TL, which value doesn't compare the treated plant length to their control, is possible to understand if a weed was shorter than the others due a higher susceptibility to the powder or parameters related to its physiology. The highest PLI was observed on *S. nigrum*, followed by *C. album* and *A. retroflexus*, while the order regarding the TL was the opposite. *A. retroflexus* was the higher weed and the less inhibited in terms of PLI, meaning that the treatments effect on the weed was not as strong as the other weeds. Instead, *C. album* and *S. nigrum* were, in this order, significantly more sensitive. Moreover, the combination of TL and PLI confirmed that pomegranate was the powder with the most allelopathic effect on the length of the weeds.

Similarly, the combination of BAI and TDM can help to understand how a treatment affects the weight of the plants. BAI, being calculated as a comparison between treated and untreated plant fresh weight, gives information about how the treatment affect the accumulation of both water and nutrient, while TDM gives information only on the percentage of nutrient into the plants. A negative BAI, and a lower TDM, suggest that the treatment promoted the weight of the plant, but most of that weight is composed by water. For instance. *A. retroflexus* was the weed with the most negative BAI, but also the lowest TDM, meaning that the treatments promoted more the accumulation of water than nutrients in its tissues. Instead, *C. album* was the opposite, meaning that the treatments didn't promote its fresh weight increase, but most of it was composed by nutrients. Regarding the powders, pomegranate gave a less negative BAI, but a higher TDM, so most of the fresh weight of the plant treated with its powder

was composed by nutrients. Instead, EP5 gave a more negative BAI, but a lower TDM, meaning that most of the weight increase promoted by the powder was due to a water accumulation into the plant. The effect of EP5 on BAI and TDM seems to be related to zeolite, as Yılmaz et al. (2014) proved that zeolite promoted water intake by the plants.

Differently from the weeds, neither weed, powders, or their interaction affected the RL of tomato plants, while the SL and the TL resulted to be slightly affected only by the presence of the weed. this result may be due to the fact that tomato plants were more competitive than weed, but also to the fact that the treatments significantly reduced the germination and growth of the weed, making them even less competitive.

Regarding the dry matter, none of the experimental factors affected tomato plants. Also in this case, as occurred for the length, this may be due to the low germination and competition of the weed after the treatments with the powders.

Regarding PLI, the slight differences observed with or without the weeds on RL, SL, and TL is not enough to justify a strong significance impact of the weed on the parameter. In this case, this incongruence may be due to physiological aspect of tomato plants and not the presence of weed. In fact, no significant differences were observed across all experimental treatments in terms of PLI.

In general, the powders showed high inhibitory potential on the selected weeds, but no phytotoxic nor promoting effect on tomato. The effects of bergamot, pomegranate, and EP5, although changing based on the studied parameter, were strong enough to cancel the competition and any possible negative effect of the weeds on tomato. Moreover, according to Conte et al. (2026a; 2026b) these powders were effective also against a wide range of fungal pathogens, included tomato ones. The different response of weed and tomato to the powder, with the first being inhibited and the second not, may be related to the timing of application. In fact, weeds were exposed to the powder since germination. Instead, regarding tomato, powders were applied on an advanced status of the plant, since they were already little seedlings. Thus, the powders are suitable for a pre-emergence application, since they are more effective on germinating roots than seedling ones. Moreover, since they do not affect grown plant root, a second cover treatment could be planned against last-emerging weeds.

5. Conclusion

In conclusion, the study proved that AWs are valid and suitable alternative for the sustainable management of the pests, especially weed, but they are also reported to be effective against fungal pathogens. Their potential is strictly linked to the timing of application, as they resulted to be more

effective of germinating roots. Based on this information, bergamot, pomegranate, and EP5, could be applied pre-emergence against early weeds, and after tomato transplant on last-emerging ones. The crop safety in cover application is related to the fact that tomato roots seem to be more resistant than weed roots. Using these AWs is favourable not only for the management of the pests, but also for human health, environment, and biodiversity. In fact, the AWs, being plant derived, are not dangerous for farmers and consumers' health, and are rapidly degraded in the soil by the microorganisms. Moreover, this usage of AWs, giving them a second life, could be a solution to their conventional management. Turn them into bio-herbicides would reduce the cost of their management, as they will get involved in circular economy processes.

The next step of the study will be to test the powder effects in open field, to ensure their efficacy also in a total uncontrolled environment.

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719 **Table 1.** Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on
720 the final germination percentage (FGP), mean germination time (MGT), germination index (GI) and
721 seed vigor index (SVI) of the weeds *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}),
722 and *Solanum nigrum* (W_{Sol}) grown on soil un-treated (P_0 or control) and treated with bergamot peel
723 powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the second *in vivo*
724 experiment.
725

49. Experimental factor	FGP (%)	MGT (days)	GI	SVI
Weed (W)				
W_{Ama}	70,8 ± 1,94 a	8,1 ± 0,1 c	2,7 ± 0,1 a	848,9 ± 33,6 a
W_{Che}	25,5 ± 2,5 b	12,4 ± 0,2 b	0,6 ± 0,1 b	277,8 ± 34,1 b
W_{Sol}	26,6 ± 1,7 b	16,6 ± 0,2 a	0,5 ± 0 b	307,2 ± 30,8 b
Powder (P)				
P_0	62,6 ± 2,1 a	12,6 ± 0,4 ab	1,8 ± 0,1 a	878,4 ± 37,1 a
P_B	29,7 ± 2,9 c	12,2 ± 0,4 ab	1 ± 0,1 c	330,9 ± 41,9 b
P_P	35,2 ± 3,3 b	12 ± 0,4 b	1,2 ± 0,1 b	325 ± 36,8 b
P_{EP5}	36,3 ± 3,8 b	12,7 ± 0,5 a	1,2 ± 0,2 b	377,6 ± 46,4 b
Weed x Powder (W x P)				
W_{Ama}-P_0	72,6 ± 5 ab	7,9 ± 0,1 d	2,8 ± 0,2 a	1063 ± 77,7 a
W_{Che}-P_0	65,7 ± 1,1 bc	13,5 ± 0,1 b	1,5 ± 0 c	812 ± 44,9 b
W_{Sol}-P_0	49,4 ± 1,8 d	16,4 ± 0,1 a	0,9 ± 0 d	760,3 ± 49 b
W_{Ama}-P_B	59,9 ± 3,6 cd	8,3 ± 0,1 d	2,3 ± 0,2 b	748,5 ± 63,6 b
W_{Che}-P_B	10,8 ± 1,8 f	12 ± 0,3 c	0,3 ± 0,1 e	86,3 ± 17,7 c
W_{Sol}-P_B	18,3 ± 1,5 ef	16,3 ± 0,3 a	0,4 ± 0 e	157,9 ± 21,2 c
W_{Ama}-P_P	70,7 ± 3,3 abc	8 ± 0,1 d	2,8 ± 0,1 a	693,5 ± 55,4 b
W_{Che}-P_P	12,2 ± 1,4 ef	11,8 ± 0,3 c	0,3 ± 0 e	109,6 ± 15,2 c
W_{Sol}-P_P	22,8 ± 2 e	16,4 ± 0,3 a	0,4 ± 0 e	171,7 ± 16,7 c
W_{Ama}-P_{EP5}	80 ± 2,2 a	8,4 ± 0,1 d	3 ± 0,1 a	890,5 ± 44,4 ab
W_{Che}-P_{EP5}	13,2 ± 1,2 ef	12,4 ± 0,5 bc	0,3 ± 0 e	103,2 ± 11,3 c
W_{Sol}-P_{EP5}	15,8 ± 2 ef	17,2 ± 0,5 a	0,3 ± 0 e	139 ± 25 c
Significance				
W	***	***	***	***
P	***	***	***	***
W x P	***	***	***	***

726 Values are means (n=16) ± standard error. In each column, means followed by the same letters are
727 not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

728 **Table 2.** Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on
729 the root, shoot, and total length (RL, SL, TL), and total dry matter (TDM) of the weeds *Amaranthus*
730 *retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}) grown on soil un-
731 treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P)
732 and EP5 commercial powder (P_{EP5}), in the second *in vivo* experiment.

Experimental factor	Length			Dry matter
	RL (cm)	SL (cm)	TL (cm)	TDM (%)
Weed (W)				
W_{Ama}	2,8 ± 0,1 b	9,1 ± 0,1 a	11,8 ± 0,1 a	18,7 ± 1,0 a
W_{Che}	3,2 ± 0,1 b	7,2 ± 0,2 b	10,3 ± 0,3 b	22,3 ± 2,3 a
W_{Sol}	4,4 ± 0,2 a	5,6 ± 0,1 c	10.0 ± 0,2 b	19,1 ± 1,7 a
Powder (P)				
P_0	5,5 ± 0,1 a	9 ± 0,1 a	14,5 ± 0,2 a	25,8 ± 2,5 a
P_B	2,3 ± 0,1 b	8,4 ± 0,2 b	10,7 ± 0,2 b	18,3 ± 2,2 b
P_P	2,6 ± 0,1 b	7 ± 0,1 c	9,6 ± 0,2 b	19,9 ± 1,8 ab
P_{EP5}	2,7 ± 0,1 b	7,9 ± 0,1 c	10,5 ± 0,2 b	16,1 ± 1,3 b
Weed x Powder (W x P)				
W_{Ama}-P_0	4,1 ± 0,1 bc	10,1 ± 0,2 a	14,2 ± 0,2 ab	25,8 ± 2,6 a
W_{Che}-P_0	5 ± 0,2 b	8,5 ± 0,3 bc	13,5 ± 0,5 bc	26,8 ± 5,5 a
W_{Sol}-P_0	8,9 ± 0,2 a	6,9 ± 0,2 d	15,8 ± 0,3 a	25 ± 4,7 a
W_{Ama}-P_B	2,1 ± 0,1 e	9,7 ± 0,2 a	11,8 ± 0,3 cd	16,3 ± 2 a
W_{Che}-P_B	2,2 ± 0,2 e	7,3 ± 0,6 cd	9,5 ± 0,7 def	23,6 ± 5,7 a
W_{Sol}-P_B	2,8 ± 0,3 de	5,6 ± 0,2 ef	8,4 ± 0,4 f	15,1 ± 2,5 a
W_{Ama}-P_P	2,6 ± 0,1 de	8,1 ± 0,1 bc	10,7 ± 0,2 de	17,1 ± 1,8 a
W_{Che}-P_P	2,5 ± 0,2 e	6,7 ± 0,4 de	9,2 ± 0,5 ef	20,3 ± 3,9 a
W_{Sol}-P_P	2,6 ± 0,1 de	4,9 ± 0,2 f	7,5 ± 0,2 f	22,3 ± 3,6 a
W_{Ama}-P_{EP5}	2,5 ± 0,1 e	8,8 ± 0,1 b	11,3 ± 0,2 d	15,8 ± 1 a
W_{Che}-P_{EP5}	2,5 ± 0,2 de	6,1 ± 0,3 def	8,6 ± 0,4 ef	18,7 ± 3,3 a
W_{Sol}-P_{EP5}	3,3 ± 0,2 cd	5,2 ± 0,3 f	8,5 ± 0,4 f	13,9 ± 1,9 a
Significance				
W	***	***	***	ns
P	***	***	***	*
W x P	***	***	***	ns

733 Values are means (n=16) ± standard error. In each column, means followed by the same letters are
734 not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

Table 3. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on the plant length inhibition (PLI) and biomass accumulation inhibition (BAI) of the weeds *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}) grown on soil untreated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the second *in vivo* experiment.

50. Experimental factor	**PLI (%)**	**BAI (%)**
Weed (W)		
W_{Ama}	15,5 ± 2,9 b	-291,9 ± 76,2 b
W_{Che}	29,4 ± 3,5 a	-60,4 ± 56,5 a
W_{Sol}	37,2 ± 3,2 a	-225,2 ± 94,9 b
Powder (P)		
P_0	0 ± 0 b	0 ± 0 a
P_B	37,2 ± 4,9 a	-325,5 ± 98,5 b
P_P	38 ± 3,1 a	-148 ± 117,3 ab
P_{EP5}	34,3 ± 3,4 a	-296,6 ± 90,7 ab
Weed x Powder (W x P)		
W_{Ama}-P_0	0 ± 0 c	0 ± 0 a
W_{Che}-P_0	0 ± 0 c	0 ± 0 a
W_{Sol}-P_0	0 ± 0 c	0 ± 0 a
W_{Ama}-P_B	10,8 ± 8,2 bc	-393,8 ± 161,8 a
W_{Che}-P_B	54,2 ± 7,7 a	-141,9 ± 78,4 a
W_{Sol}-P_B	46,6 ± 7,2 a	-440,8 ± 235,1 a
W_{Ama}-P_P	30 ± 5,4 ab	-187,1 ± 76,7 a
W_{Che}-P_P	30,2 ± 5,9 ab	69,3 ± 201,8 a
W_{Sol}-P_P	53,7 ± 3 a	-326 ± 278,1 a
W_{Ama}-P_{EP5}	21,1 ± 4,6 bc	-586,7 ± 235,7 a
W_{Che}-P_{EP5}	33,4 ± 6,2 ab	-169,1 ± 64,2 a
W_{Sol}-P_{EP5}	48,5 ± 5,6 a	-134,1 ± 104,8 a
Significance		
W	***	***
P	***	***
W x P	***	***

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

Table 4. Effect of the factors Weed (W), Powder (P) and the interaction Weed x Powder (W x P) on the root (RL), shoot (SL) and total length (TL), root (RDM), shoot (SDM) and total dry matter content (TDM) of tomato plants grown on soil weed-free (W_0 or control) and infested with *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}) soil, un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the *in vivo* experiment.

Experimental factor	RL	SL	TL	RDM	SDM	TDM
	(cm)			(% fw)		
Weed (W)						
W_0	20,7 ± 0,5 a	56,4 ± 1,1 a	77,1 ± 1,3 a	21,8 ± 0,7 a	17,4 ± 0,7 a	17,9 ± 0,6 a
W_{Ama}	19,0 ± 0,7 a	50,1 ± 1,1 b	69,1 ± 1,5 b	22 ± 1 a	20,4 ± 1,5 a	20,1 ± 1,1 a
W_{Che}	19,9 ± 0,6 a	52,2 ± 0,8 b	72,1 ± 1,2 ab	23,4 ± 1,2 a	19,2 ± 1,3 a	19,3 ± 1 a
W_{Sol}	19,4 ± 0,8 a	48,4 ± 1,3 b	67,7 ± 1,7 b	23,4 ± 1,4 a	19,9 ± 1,4 a	19,4 ± 1 a
Powder (P)						
P_0	20,1 ± 0,7 a	55,2 ± 1,2 a	75,3 ± 1,5 a	24,9 ± 1,1 a	18,8 ± 1,2 a	25,85 ± 2,53 a
P_B	18,4 ± 0,9 a	50,8 ± 1,2 a	69,1 ± 1,8 a	21 ± 1,3 a	22 ± 2 a	18,3 ± 2,19 a
P_P	21,2 ± 0,6 a	50 ± 0,9 a	71,3 ± 1,1 a	22 ± 0,9 a	17,9 ± 0,9 a	19,87 ± 1,84 a
P_{EP5}	19,3 ± 0,5 a	51 ± 1,1 a	70,3 ± 1,4 a	22,6 ± 1 a	18,2 ± 0,7 a	16,13 ± 1,31 a
Weed x Powder (W x P)						
W_0-P_0	20,8 ± 1,3 a	63,5 ± 1,6 a	84,4 ± 1,9 a	24,2 ± 1,7 a	16,4 ± 0,3 a	17,3 ± 0,4 a
W_{Ama}-P_0	20,2 ± 1,9 a	52,5 ± 2,6 a	72,7 ± 3,2 a	25 ± 1,7 a	23,5 ± 3,9 a	21,6 ± 2 a
W_{Che}-P_0	19,4 ± 1,3 a	54 ± 1,7 a	73,5 ± 2,6 a	25,3 ± 2,5 a	18,9 ± 2,3 a	19,7 ± 2,1 a
W_{Sol}-P_0	19,8 ± 1,0 a	50,8 ± 2,8 a	70,6 ± 3,6 a	25 ± 2,5 a	16,5 ± 0,5 a	18,6 ± 1,1 a
W_0-P_B	19,9 ± 0,9 a	54,8 ± 2 a	74,6 ± 2,7 a	19,8 ± 0,9 a	16,7 ± 1,1 a	16,9 ± 0,7 a
W_{Ama}-P_B	17,8 ± 1,8 a	49,3 ± 2,8 a	67,1 ± 4,4 a	18,9 ± 2,7 a	23,9 ± 4,5 a	23,4 ± 3,9 a
W_{Che}-P_B	17,2 ± 1,2 a	51 ± 2,1 a	68,2 ± 3,1 a	24,3 ± 3 a	23 ± 4,5 a	21,4 ± 2,9 a
W_{Sol}-P_B	18,6 ± 2,6 a	48 ± 2,6 a	66,6 ± 3,6 a	21,2 ± 3,3 a	24,4 ± 4,6 a	20,5 ± 3 a
W_0-P_P	21,4 ± 1,0 a	51,7 ± 2,1 a	73,1 ± 2,4 a	21,1 ± 1,5 a	17 ± 0,9 a	17,6 ± 0,9 a
W_{Ama}-P_P	20,3 ± 0,9 a	49,5 ± 1,5 a	69,8 ± 2,1 a	25 ± 2,2 a	17,9 ± 0,6 a	18,8 ± 0,7 a
W_{Che}-P_P	22,0 ± 1,2 a	51,9 ± 1,2 a	73,9 ± 1,8 a	21,5 ± 1,9 a	16,6 ± 1,5 a	17,3 ± 1,5 a
W_{Sol}-P_P	21,3 ± 1,5 a	46,9 ± 2 a	68,3 ± 2,3 a	20,6 ± 1,9 a	20,1 ± 2,9 a	18,9 ± 1,8 a
W_0-P_{EP5}	20,8 ± 1,2 a	55,5 ± 2 a	76,3 ± 2,4 a	22 ± 1 a	19,3 ± 2,1 a	19,9 ± 2 a
W_{Ama}-P_{EP5}	17,6 ± 1,1 a	49,2 ± 1,5 a	66,8 ± 2,1 a	19,2 ± 0,9 a	16,2 ± 0,9 a	16,5 ± 0,9 a
W_{Che}-P_{EP5}	21,0 ± 0,6 a	51,7 ± 1,4 a	72,7 ± 1,5 a	22,4 ± 1,7 a	18,4 ± 0,6 a	18,9 ± 0,6 a
W_{Sol}-P_{EP5}	17,7 ± 1,1 a	47,7 ± 2,9 a	65,4 ± 3,8 a	26,8 ± 3,3 a	18,8 ± 1,1 a	19,7 ± 1,2 a
Significance						
W	ns	*	*	ns	ns	ns
P	ns	ns	ns	ns	ns	ns
W x P	ns	ns	ns	ns	ns	ns

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test).
***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

Table 5. Effect of the factors Species (S), Weed (W) and the interaction Weed x Extract (W x E) on plant length inhibition (PLI) and biomass accumulation index (BAI) of tomato plants grown on soil weed-free (W_0 or control) and infested with *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}) soil, un-treated (P_0 or control) and treated with bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}), in the *in vivo* experiment.

Experimental factor	PLI (%)	BAI (%)
Weed (W)		
W_0	7,8 ± 1,6 b	3,2 ± 4 a
W_{Ama}	17,3 ± 2 a	17,9 ± 4,2 a
W_{Che}	13,5 ± 1,7 ab	10 ± 4,6 a
W_{Sol}	18,5 ± 2,2 a	18,1 ± 4,7 a
Powder (P)		
P_0	10 ± 1,8 a	7 ± 3,6 a
P_B	17 ± 2,3 a	14,1 ± 5 a
P_P	14,7 ± 1,5 a	18,1 ± 4,6 a
P_{EP5}	15,4 ± 2 a	10 ± 4,4 a
Weed x Powder (W x P)		
W_0-P_0	0 ± 0 a	0 ± 0 a
W_{Ama}-P_0	13 ± 3,9 a	17,1 ± 7,7 a
W_{Che}-P_0	12 ± 3,4 a	6,9 ± 7,9 a
W_{Sol}-P_0	14,9 ± 4,5 a	4,1 ± 8,9 a
W_0-P_B	10,7 ± 3,4 a	-2,4 ± 10,1 a
W_{Ama}-P_B	19,7 ± 5,7 a	17,5 ± 10,6 a
W_{Che}-P_B	17,7 ± 4,3 a	21 ± 8,8 a
W_{Sol}-P_B	20 ± 4,6 a	20,1 ± 9,9 a
W_0-P_P	12,3 ± 3,5 a	20,7 ± 6,7 a
W_{Ama}-P_P	16,7 ± 2,6 a	23,6 ± 7,6 a
W_{Che}-P_P	11,7 ± 2,4 a	2,3 ± 12,2 a
W_{Sol}-P_P	18,1 ± 3,4 a	25,8 ± 8,8 a
W_0-P_{EP5}	8,3 ± 3,6 a	-5,6 ± 9,7 a
W_{Ama}-P_{EP5}	19,8 ± 3,1 a	13,6 ± 7,7 a
W_{Che}-P_{EP5}	12,7 ± 2,8 a	9,6 ± 7 a
W_{Sol}-P_{EP5}	21 ± 5,3 a	22,3 ± 9,8 a
Significance		
W	***	ns
P	ns	ns
W x P	ns	ns

Values are means (n=16) ± standard error. In each column, means followed by the same letters are not significantly different ($p \leq 0.05$; Tukey's test). ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

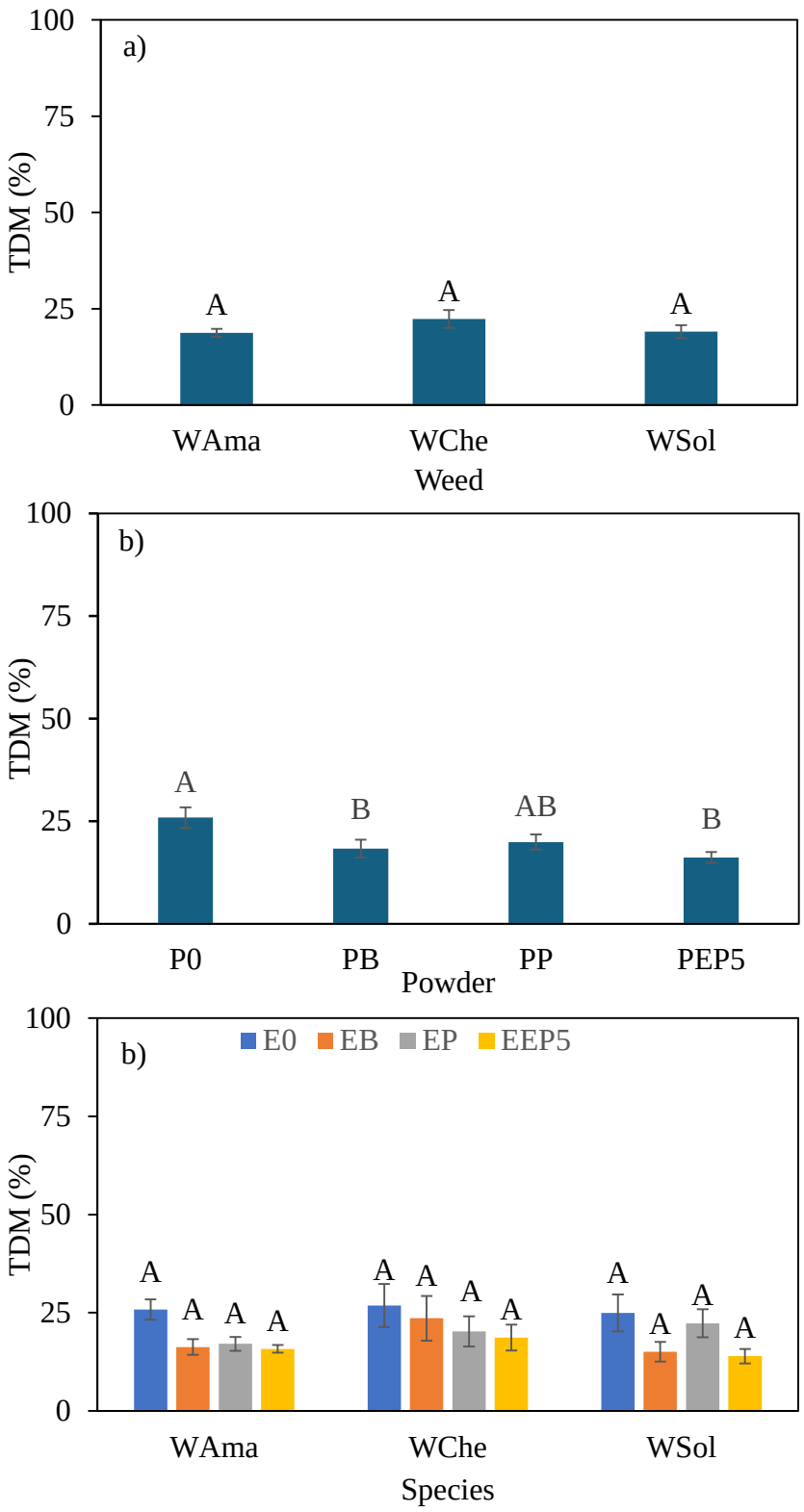


Figure 1. Total dry matter (TDM) of weeds in the in vivo experiment, as affected by: a) the factor Weed (W); Amaranthus retroflexus (WAma), Chenopodium album (WChe), and Solanum nigrum (WSol); b) the factor Powder (P); un-treated (P0 or control) bergamot peel powder (PB), pomegranate peel powder (PP) and EP5 commercial powder (PEP5); c) the interaction W x F. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

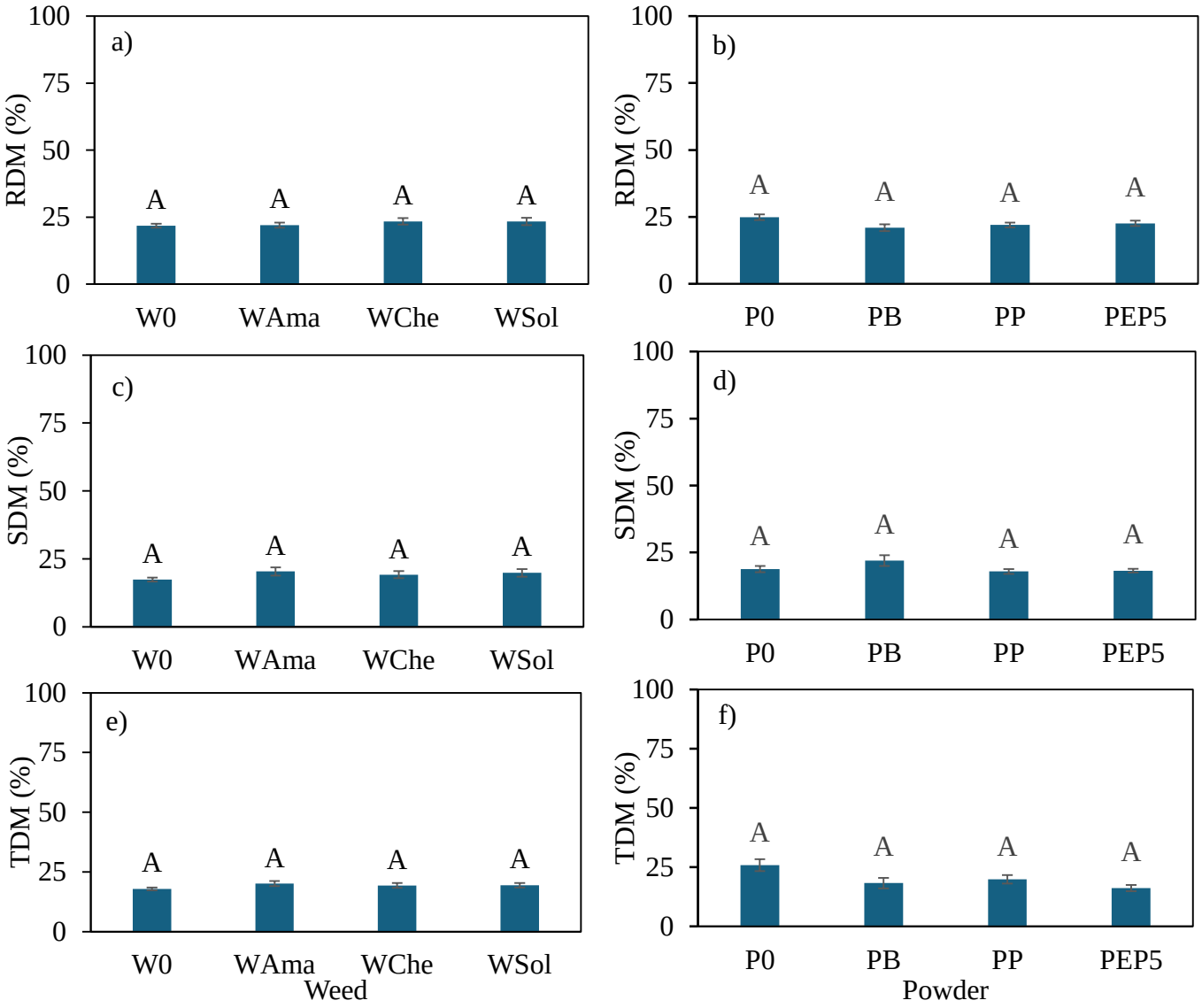


Figure 2. Dry matter of tomato in the *in vivo* experiment: a) root dry matter (RDM) affected by the factor Weed (W); soil weed-free (W_0 or control), *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}); b) root dry matter (RDM) affected by the factor Powder (P); un-treated (P_0 or control) bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}); c) shoot dry matter (SDM) affected by the factor Weed (W); soil weed-free (W_0 or control), *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}); d) shoot dry matter (SDM) affected by the factor Powder (P); un-treated (P_0 or control) bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}); e) total dry matter (TDM) affected by the factor Weed (W); soil weed-free (W_0 or control), *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}); f) total dry matter (RDM) affected by the factor Powder (P); un-treated (P_0 or control) bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}). Values are means (n=4) ± standard error. Different letters above bars indicate significant differences among species and extracts ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

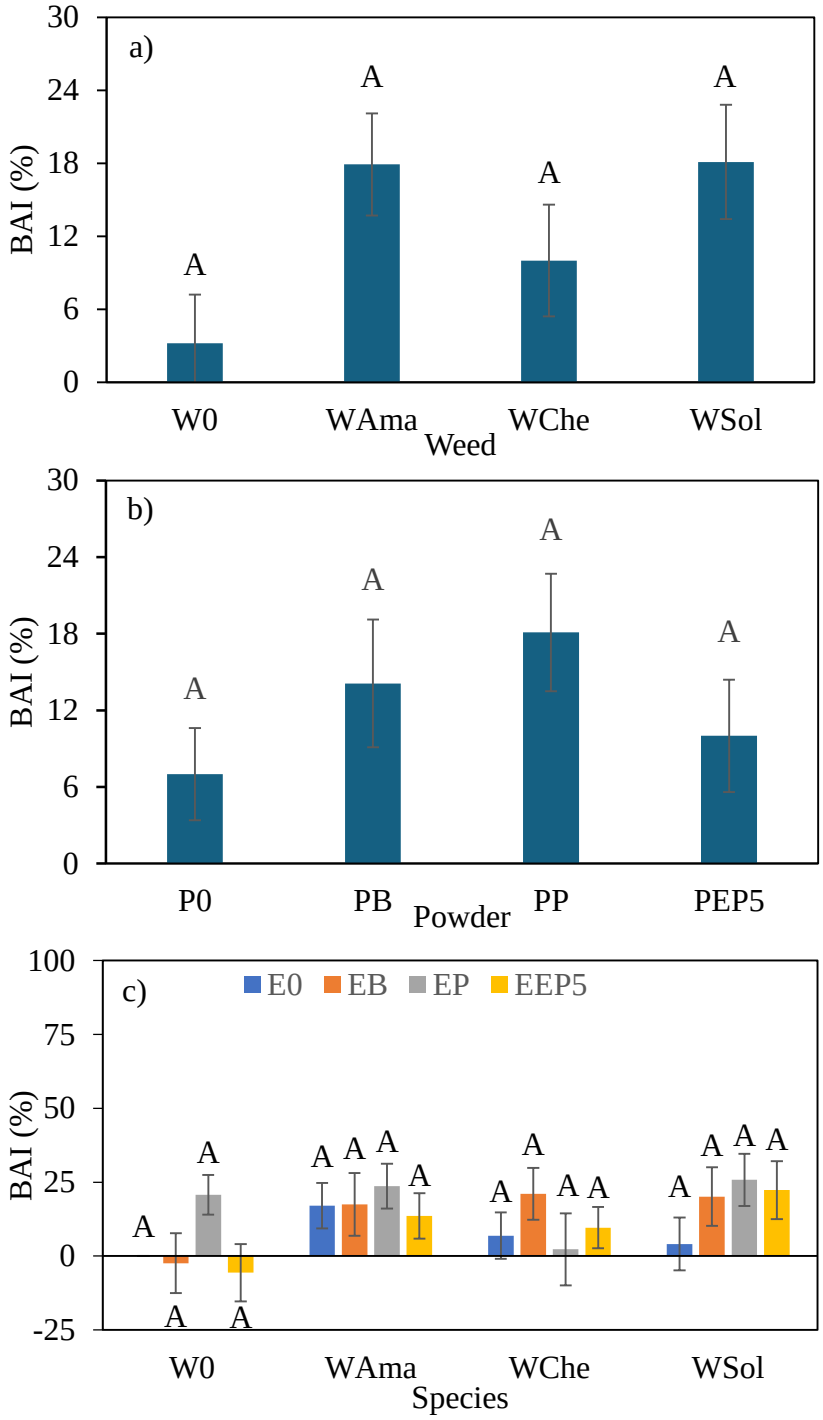


Figure 3. Biomass accumulation inhibition (BAI) of tomato in the *in vivo* experiment, as affected by: a) the factor Weed (W); soil weed-free (W_0 or control), *Amaranthus retroflexus* (W_{Ama}), *Chenopodium album* (W_{Che}), and *Solanum nigrum* (W_{Sol}); b) the factor Powder (P); un-treated (P_0 or control) bergamot peel powder (P_B), pomegranate peel powder (P_P) and EP5 commercial powder (P_{EP5}); c) the interaction W x F. Values are means (n=16) ± standard error. Different letters above bars indicate significant differences among species and powders ($p \leq 0.05$; Tukey's test). Uppercase letters refer to F test significance at $p < 0.001$.

Table S1. Treatment combinations used in the *in vivo* experiment.

Weed (W)	Powder (P)	Abbreviation
-	-	W_0-P_0 (control)
A. retroflexsus	-	W_{Ama}-P_0
C. album	-	W_{Che}-P_0
S. niger	-	W_{Sol}-P_0
-	Bergamot	W_0-P_B
A. retroflexsus	Bergamot	W_{Ama}-P_B
C. album	Bergamot	W_{Che}-P_B
S. niger	Bergamot	W_{Sol}-P_B
-	Pomegranate	W_0-P_P
A. retroflexsus	Pomegranate	W_{Ama}-P_P
C. album	Pomegranate	W_{Che}-P_P
S. niger	Pomegranate	W_{Sol}-P_P
-	EP5	W_0-P_{EP5}
A. retroflexsus	EP5	W_{Ama}-P_{EP5}
C. album	EP5	W_{Che}-P_{EP5}
S. niger	EP5	W_{Sol}-P_{EP5}

Objective 6. Evaluation of three *Streptomyces* strains (Strep_22, Strep_23, and Strep_24) to inhibit fungal soilborne pathogens under *in vitro* conditions, as well as the in vivo efficacy of the best performer *Strepmotyces* strain (Strep_22) *in vivo* against the soilborne fungal pathogens of wheat and tomato.

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Research Topics

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Research Topics

Plant pathology

Biocontrol and plant growth-promoting potential of *Streptomyces* strains against fungal pathogens of tomato and wheat for a sustainable agriculture

Running title

Streptomyces biocontrol for sustainable wheat and tomato agriculture

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Abstract

BACKGROUND: Modern agriculture urgently requires sustainable alternatives to synthetic chemical pesticides to mitigate crop yield losses while protecting human health and the environment. This study evaluated the biocontrol potential and plant growth-promoting (PGP) traits of three newly isolated *Streptomyces* strains (Strep_22, Strep_23, and Strep_24) against severe soilborne fungal pathogens affecting durum wheat (*Triticum durum*) and tomato (*Solanum lycopersicum*), two economically crucial crops.

RESULTS: In *in vitro* dual-culture assays, strain Strep_22 demonstrated the highest overall efficacy, achieving a mean fungal growth inhibition rate of 91.52% and near-complete suppression of critical pathogens such as *Agroathelia rolfsii, Fusarium solani*, and *Sclerotinia sclerotiorum*. Kinetic modelling confirmed that Strep_22 significantly reduced fungal growth rates. Based on these outstanding findings, Strep_22 was selected for in vivo greenhouse validation. Greenhouse trials demonstrated that treatments with this strain significantly reduced disease incidence and severity (such as root rot and wilt) in both wheat and tomato crops. Concurrently, Strep_22 exhibited strong PGP traits, inducing a significant increase in plant biomass, shoot length, and root development compared to untreated, infected controls.

CONCLUSION: The *Streptomyces* strain Strep_22 represents a highly promising, dual-action biocontrol agent. It effectively protects wheat and tomato crops against devastating fungal infections while simultaneously stimulating plant vegetative growth and development. Consequently, it constitutes a valuable ecological alternative for the formulation of commercial biofertilizers and biopesticides, paving the way toward sustainable agricultural systems.

Keywords: Biocontrol, *Streptomyces* spp., Soilborne fungal pathogens, *Triticum durum, Solanum lycopersicum,* Plant growth-promoting bacteria (PGPB).

1 INTRODUCTION

Agricultural losses carry severe social, economic, healthcare, and environmental implications. Production-boosting practices are ineffective unless paired with strategies to minimize these losses. Understanding the influencing factors, geographic contexts, and damage types is key to developing effective control strategies.[1] According to FAO, pests, including pathogens, weeds, and insects, severely affect 20–40% of global crops.[2] Without protection, potential losses reach 75%, driven by weeds (34%), animal pests (18%), and microbial pathogens (16%).[3] As major global crops, wheat and tomato suffer substantial yield losses from fungal, bacterial, and viral pathogens, and insect infestations. Pests and diseases cause an average global yield loss of 21.5% in wheat and 25–40% in tomato, depending on the region and viral outbreaks.[2,4] These losses are exacerbated by emerging diseases interacting with climate instability. In Italy, durum wheat (*Triticum durum* Desf.) yields fluctuate by 10–15% due to fungal pathogens combined with prolonged spring droughts.[5] For Italian tomato crops, direct yield losses reach 15–22%, while indirect quality depreciation accounts for 10–12%.[5] Managing these damages increases agrochemical costs, triggering environmental and health degradation.[6] In 2025, Italian wheat production totalled 4,3 million tonnes, with durum wheat exceeding 55%.[7] This production is concentrated in Southern Italy, particularly in Apulia, which represents 29% of the national cultivated area and 24% of the total yield.[8] Italy is also the leading European tomato producer, contributing 36% of total EU volume in 2025 (5,8 million tonnes), with Apulia providing 22.3% of the national yield.[9,10] For wheat, potential losses from pathogens, pests, and weeds are 18%, 9%, and 23%, reducible via crop protection to 13%, 8%, and 8%, respectively.[3] Conversely, tomato crops exhibit higher vulnerability to fungal pathogens, with potential yield losses of 51–53%.[11] Identifying primary biotic threats is essential to limit these losses sustainably. Fungal pathogens threatening these crops are highly diverse. Wheat yield is primarily

affected by *Fusarium* spp. (*F. avenaceum*, *F. culmorum*, *F. oxysporum*, and *F. graminearum*).[12] Tomato crops are threatened by a wider range of fungal diseases: southern blight (*Agroathelia rolfsii*), early blight (*Alternaria solani*), anthracnose (*Colletotrichum* spp.), fusarium wilt (*F. oxysporum* f.sp. *lycopersici*), and verticillium wilt (*Verticillium albo-atrum* and *Verticillium dahliae*).[13,14] Chemical management poses risks to biodiversity and human health, including antibiotic resistance and neurological disorders, driving the need for sustainable practices.[15] Consequently, interest in biological control agents (BCAs) like *Trichoderma* spp. and *Bacillus* spp. has grown significantly. *Trichoderma* spp. inhibit the mycelial growth of various pathogens, including *A. solani*, *Fusarium* spp., *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*.[16-19] Its efficacy relies on mycoparasitism, antibiosis, and competition for space and nutrients.[20] Similarly, *Bacillus* spp. produce antimicrobial compounds, compete for resources, and induce systemic resistance (ISR) in host plants, controlling *A. solani*, *F. graminearum*, and *F. oxysporum*.[17,18,21] Recently, *Streptomyces* spp. have gained attention as high-potential BCAs.[22-24] *Streptomyces* is the largest genus among *Actinomycetes*, with over 900 species widespread in terrestrial and aquatic environments.[25,26] Primarily saprophytic decomposers, most are beneficial endosymbionts forming close bonds with plant root).[27] Their biocontrol activity relies on synthesizing biomolecules active against numerous phytopathogens.[28] Their filamentous, sporulating structures and production of chitinases and cellulases degrade competitor cell walls and facilitate rhizosphere colonization.[29-30] Furthermore, they synthesize bioactive secondary metabolites that constitute 70–80% of natural products used in pharmacology and agrochemicals.[31] These bacteria act as plant growth-promoting rhizobacteria (PGPR) by producing auxins and assisting in nutrient acquisition via phosphate solubilization and siderophore production.[26] Most antibiotic-producing actinomycetes belong to this genus, driving commercial interest.[32] The bioactivity of *Streptomyces* is linked to environmental interactions; for instance, antibiotic production is often triggered by microbial competitors.[31,33] Localized antibiotic production increases significantly during interactions with other microorganisms.[34,35] Moreover, these endosymbionts activate induced systemic resistance (ISR) in plants, enhancing immunity against pests and diseases.[26,36]

Based on relevant interest about *Strepmtomyces* genus, the present study aims to: a) evaluate and compare the *in vitro* biocontrol potential of specific *Streptomyces* strains against some severe soilborne fungal pathogens of wheat and tomato crops; b) assess the *in vivo* antifungal properties of the most effective *Streptomyces* strain by setting greenhouse experiments; c) and analyse the beneficial effects of potential of these strains on the growth and development of wheat and tomato crops.

2 MATERIAL AND METHODS

2.1 Isolation and identification of Streptomyces strains

During surveys carried out from 2022 to 2024 on the rhizosphere of various plant hosts, *Actinomycetes* and other microorganisms were isolated. Three microbial cultures resembling as *Actinomycetes* (Strep_22, Strep_23 and Strep_24) were purified following the method described by[23] in order to perform molecular identification and characterization. The DNA extraction was carried out according to Carlucci et al. (2013), and the amplification using primer pairs 16SAct1F (5′CGC GGC CTA TCA GCT TGT TG 3′) and 16SAct1R (5′ CCG TAC TCC CCA GGC GGGG 3′) of 16S ribosomial rDNA region according.[23]

2.2 Fungal soilborne pathogens of wheat and tomato crops

To assay the ability of *Streptomyces* strains (Strep_22, Strep_23, and Strep_24) to inhibit fungal growth, ten economically relevant soilborne pathogens were selected from the collection of the Plant Pathology and Diagnostic Laboratory at the University of Foggia (DAFNE), Italy. These isolates, collected from symptomatic crops in the Foggia province, included: *Agroathelia rolfsii* (from tomato; Aro), *Fusarium algeriense* (wheat; Fal), *F. avenaceum* (tomato; Fav), *F. culmorum* (wheat; Fcu), *F. oxysporum* (tomato; Fox), *F. oxysporum* f. sp. *dianthi* (wheat; Fox_d), *F. solani* (tomato; Fsol), *Rhizoctonia solani* (grapevine; Rso), *Sclerotinia sclerotiorum* (broccoli; Ssc), and *Verticillium dahliae* (olive tree; Vda). These specific pathogens were previously evaluated with plant-derived products by Morea et al.[37] and Conte et al.[10,38]. Morphological and molecular taxonomic identification followed the DNA extraction protocol by Carlucci et al.[39], as detailed by Morea et al.[37]

2.3 Inhibitory Activity in dual cultures

Dual culture assays were performed by modifying the protocol of Carlucci et al. (2022). A 4 mm agar-mycelium disc from a 21-day-old *Streptomyces* colony grown on PDA was placed face-down on a fresh PDA Petri dish, 1.0 cm from the centre. Plates were sealed with Parafilm and incubated at 25 ± 3 °C in darkness. After 10 days, a 4 mm agar disc of the target fungal pathogen was placed 1.0 cm from the centre, directly opposite the *Streptomyces* disc. Plates were re-sealed and incubated under the same conditions until the fungal

pathogens in the control plates, consisting of the pathogen opposite a sterile agar disc, reached maximum growth, or up to 21 days for slow-growing isolates. The experiment was conducted with two replicates per strain–pathogen combination. The inhibitory activity (IA; %) was calculated using the following formula:

$$IA\ (\%) = \frac{R_1 - R_2}{R_1} \times 100$$

where R_1 was the radius measurement from the centre of the colony of fungal pathogen towards the edge of the control Petri plate, and R_2 was the radius measurement from the centre of the colony of fungal pathogen toward the *Streptomyces* colony.

2.4 Growth rates and kinetics of the fungal pathogens

Radial growth measurements were recorded every 48 hours. These growth rates were used to determine the growth kinetics using the modified logistic equation described by Dantigny et al.[40], as shown below:

$$D = \frac{D_{max}}{1 + exp[k(\tau - t)]}$$

where D is the diameter of the colony over time; D_{max} is the maximum diameter of the fungal colony (55 mm); k is the rate of fungal growth (mm/hour); τ is the time to attain a half of D_{max} (hours) and t is the time (hours).

The Dantigny function, modified based on Bevilacqua et al.[41], was used to model the radial growth of fungal colonies. The maximum growth extent was set to 55 mm (the plate diameter). The fitting procedure estimated the growth rate (diameter increase per 24 hours) and the parameter τ (the time in hours required to reach a 27.5 mm diameter). The Dantigny equation satisfactorily fitted all data sets, yielding regression coefficients between 0.909 and 0.999. Following this, secondary modelling was performed as described by Bevilacqua et al. (2017), where τ was standardized as:

$$\Delta\,\tau = \tau_{Strep} - \tau_C$$

where τ_{Strep} is the inhibition value due to *Streptomyces* strain presence in dual culture against fungal growth, and τ_C is the value obtained in the control plate of fungal species without any *Streptomyces* strain.

2.5 Evaluation of inhibitory activity by Streptomyces sp. in greenhouse condition

Based on *in vitro* results, Strep_22 was selected for greenhouse experiments on wheat and tomato to evaluate its plant growth-promoting (PGP) potential and protective efficacy. Two specific pathogen pools were used: *F. algeriense*, *F. culmorum*, and *F. oxysporum* f. sp. *dianthi* for wheat; and *A. rolfsii*, *F. oxysporum*, and *S. sclerotiorum* for tomato.

2.5.1 Assessment of native fungal infections on wheat kernels and tomato seedling

Before greenhouse experiments, wheat kernels (variety RGT Voilur) and tomato seedlings (variety Taylor) underwent mycological analysis using the isolation technique of Fisher et al.[42]. One hundred surface-disinfected wheat kernels were incubated on PDA at 25 ± 3°C for 14 days. Similarly, five tissue fragments from the root, collar, and basal stem of twenty tomato seedlings were plated. Developing fungal isolates were microscopically examined to determine genus and, where possible, species.

2.5.2 Artificial fungal inoculum preparation for wheat and tomato experiments

The artificial fungal inoculum for both experiments followed the method of Conte et al.[10,38]. Glass jars containing 100 g of wheat kernels were soaked in tap water for 12 h, drained, and autoclaved three times at 180°C for 20 min. The sterilized kernels were then inoculated with 5 mL of a fungal propagule suspension (10^5 CFU mL^{-1}), prepared by scraping 14-day-old colonies into sterile distilled water with 0.5% Tween 20. Jars were sealed and incubated in the dark at 25 ± 3 °C for 21 days.

2.5.3 Setup of the greenhouse experiments

Greenhouse experiments for wheat (February–April 2024) and tomato (May–July 2024) were conducted at DAFNE, University of Foggia. In 1.5 L pots lined with non-woven fabric, 5 cm of Gaia soil was added. Twelve pathogen-inoculated kernels were placed on the soil, covered with 1.0 cm of soil, watered with 150 mL of water, and sealed with plastic film. After 72 h, 40.0 mL of Strep_22 inoculum (10^7 CFU mL^{-1} in 0.2% Tween 20, prepared per Carlucci et al.[23]) was added, covered with 1.0 cm of soil, and resealed. After another 72 h, thirty wheat kernels or one tomato seedling were planted per pot and covered with 1.0 cm of soil. Both experiments comprised eight trials (including mocks and negative controls), with 16 replicates for wheat and 24 for tomato.

2.6 In in vivo Assessment of Strep_22 as biocontrol agent against fungal pathogens on wheat and tomato

In the wheat experiment, seed germination rates were recorded every 48 h for 10 days. Afterward, plant phytosanitary status—specifically leaf yellowing and root symptoms—was assessed using an empirical seven-class scale (0: no symptoms; 1: 1%–15%; 2: 16%–35%; 3: 36%–50%; 4: 51%–70%; 5: 71%–85%; 6: 86%–100% infection). Root disease incidence was then calculated as the McKinney Index (MkI; %) using the following formula[43]:

$$MkI\ (\%) = \frac{\sum(N_n \times C_n)}{N_{tot} \times C_{max}} \times 100$$

where N_n represents the number of cases observed for each class, C_n is the reference class, N_{tot} is the total number of cases observed, and C_{max} is the highest class of the scale.

After 45 days, tomato plants were gently removed and their roots washed. Root status was assessed using the same seven-class empirical scale as the wheat experiment. Damage to the aerial parts and collar was evaluated using two descriptive six-class scales: aerial parts (0: healthy; 1: slightly yellow; 2: completely yellow; 3: slightly necrotized; 4: completely necrotized; 5: dead) and collar (0: healthy; 1: hydropic tissues; 2: brown; 3: slightly rotten; 4: rotten; 5: heavily rotten).

2.7 Plant Growth Promotion performed by Strep_22

After 90 days for wheat and 45 days for tomato, plants were removed from the soil, their roots washed, and total height (cm) and fresh weight (g) measured. Each plant was then dried in a forced-air oven at 30.0 °C until a constant weight was reached. Once completely dehydrated, the dry matter content was calculated using the following formula:

$$Dry - matter = \frac{Dry\ sample\ weight\ (g)}{Fresh\ sample\ weight\ (g)} \times 100$$

2.8 Re-Isolation of artificially inoculated fungal pathogens

Prior to oven-drying, several root portions were set aside for mycological analysis to confirm the presence of the inoculated fungi. This was quantified as the re-isolation frequency (R-IF; %), which was calculated according to the following formula:

$$R - IF\ (\%) = \left(\frac{n_i}{N_p}\right) \times 100$$

where n_i is the number of root segments showing growth of the inoculated fungal pathogen; and N_p is the total number of root segments plated.

Roots from each plant were washed with distilled water and cut into 1.0 cm segments. These tissue segments were surface-disinfected following the method of Fisher et al.[42] For each trial, six root pieces were placed onto PDA amended with 500 ppm of streptomycin, using two plates per trial. After a 21-day incubation period, the resulting fungal colonies underwent morphological and microscopic examination to confirm the identity of the artificially inoculated fungi.

2.9 Statistical Analyses

Statistical analyses for all experiments were performed using JMP software (version 16.2). Percentage data for fungal growth inhibition (IA) and disease control (MkI) were arcsine square-root transformed (Degrees(arcsin($\sqrt{X}$))). The Shapiro–Wilk and Levene tests assessed data normality and homogeneity of variance, respectively. Fungal growth kinetics were fitted via the least-squares approach using Statistica for Windows (version 12.0). For *in vitro* trials, one-way ANOVA determined significant differences in IA across the ten fungal species and three *Streptomyces* strains. For greenhouse data (collected January 2024–August 2025), factorial ANOVA evaluated the significance of Strep_22 efficacy, fungal sensitivity, and their interactions (fungus × Strep_22) on IA and MkI. Plant height and dry matter were similarly analyzed using one-way and factorial ANOVAs. Treatment means were compared using Tukey's HSD test ($p < 0.01$).

3 RESULTS

3.1 Isolation and identification of Streptomyces strains

The taxonomic identification of the *Streptomyces* strain Strep_22, performed using the primers described by Carlucci et al.[23], was not successful because the BLAST sequence identity was below 95% and the results were ambiguous. This result suggests that this actinomycetous microorganism warrants a more detailed genomic study, involving whole-genome sequencing and bioinformatic analysis (data not shown).

3.2 In vitro inhibition performed by Streptomyces sp. against soilborne fungal pathogens

Fig. 1 shows the dual culture assay results. Panel (a) illustrates the overall efficacy of individual *Streptomyces* strains against the soilborne pathogens: strain Strep_22 was the most effective (IA = 91.52%), followed by Strep_24 (IA = 85.61%), while Strep_23 was the least effective (IA = 34.64%). Panel (b) highlights pathogen sensitivity: *V. dahliae* and *F. avenaceum* were the most sensitive, with IA values of 89.40% and 87.58%, respectively. Conversely, *F. culmorum* (IA = 59.55%) and *R. solani* (IA = 47.57%) showed the highest resistance. The remaining pathogens were moderately sensitive: *F. oxysporum* (IA = 78.94%), *F. algeriense* (IA = 78.03%), *F. oxysporum* f.sp. *dianthi* (IA = 68.79%), *F. solani* (IA = 68.18%), *S. sclerotiorum* (IA = 66.67%), and *A. rolfsii* (IA = 61.21%).

Fig. 2 and 3 show the specific sensitivity of each pathogen to individual *Streptomyces* strains. Strep_22 achieved near-total inhibition against almost all isolates, exceeding 85.00% IA for all pathogens except *R. solani* (55.45%), and reaching 100.00% against *A. rolfsii*, *F. solani*, and *S. sclerotiorum* (Panel a). In contrast, Strep_23 showed notable activity against only a few pathogens, such as *V. dahliae* (82.73%), *F. avenaceum* (74.55%), *F. algeriense* (63.64%), *F. oxysporum* (55.45%), and *F. oxysporum* f.sp. *dianthi* (55.45%), while *F. solani* was largely unaffected (12.73%) and the rest showed no sensitivity (Panel b). Strep_24 also performed remarkably well, with IA values ranging from 56.36% to 100.00% (Panel c). Although slightly less broad-spectrum than Strep_22, its activity was more species-specific, performing better against *F. culmorum* (89.55%), *V. dahliae* (94.55%), and *R. solani* (87.27%).

Fig. 4 confirms that strain Strep_22 was the most effective inhibitor, reducing final colony size *in vitro* and slowing fungal growth rates. This is evidenced by a significant increase in the time required to reach half-maximum dimension ($\Delta\tau$ = 101.84 hours), with inhibition maintained throughout the 440-hour experiment. Conversely, Strep_23 and Strep_24 showed negligible changes in this parameter compared to control plates (-5.21 and -5.32 hours, respectively). While Strep_23 kinetics reflect its low IA, data for Strep_24 suggest pathogens only slowed upon nearing the strain, growing at control-plate speed during the initial days. Among the pathogens, *S. sclerotiorum* ($\Delta\tau$ = 212.80 hours), *F. solani* ($\Delta\tau$ = 108.74 hours), and *A. rolfsii* ($\Delta\tau$ = 105.46 hours) exhibited the highest growth rate inhibition. In contrast, *F. culmorum* showed almost no growth rate inhibition ($\Delta\tau$ = 7.44 hours), while the remaining isolates (*R. solani*, *F. avenaceum*, *F. oxysporum*, *F. oxysporum* f.sp. *dianthi*, *F. algeriense*, and *V. dahliae*) appeared promoted in early stages. Interestingly, *V. dahliae* was the most sensitive in final growth reduction but the least sensitive in initial growth speed, indicating *Streptomyces* strains reduce its growth rate primarily during the second half of its growth phase.

3.3 Assessment of Streptomyces strain Strep_22 as putative antagonists against fungal phytopathogens affecting wheat and tomato plants in greenhouse

3.3.1 Mycological analyses on wheat kernels and tomato seedlings

Fungal isolation revealed that almost all wheat kernels were infected by endogenous pathogens, with *Alternaria* and *Fusarium* being the most abundant, while only a small number remained un-infected. In contrast, the tomato roots were significantly healthier, displaying very low isolation rates of fungal pathogens (*Alternaria* spp., 3%; *Fusarium* spp., 5%).

3.3.2 Evaluation of the antifungal activity of strain Strep_22 against fungal phytopathogens in wheat

Fig. 5 shows the final germination percentage of wheat kernels, indicating that neither the fungal pathogens nor strain Strep_22 significantly affected germination compared to the un-treated control (all values around 90–91%). Regarding the phytosanitary status of wheat roots, Fig. 6 confirms the *in vivo* efficacy of Strep_22. Among the pathogens, *F. algeriense* was the most aggressive (MkI = 57.43%), followed by *F. oxysporum* f.sp. *dianthi* (47.86%) and *F. culmorum* (42.27%). Notably, treatment with Strep_22 significantly reduced the mean root infection index (MkI) from 68.80% in un-treated trials to 19.64%. Data regarding the interaction between Strep_22 and individual pathogens revealed that treatment efficacy varied by target. The strain was highly effective at reducing infections caused by *F. culmorum* (MkI dropped from 69.60% to 14.94%) and *F. oxysporum* f.sp. *dianthi* (from 79.06% to 19.65%), compared to *F. algeriense* (from 81.76% to 33.09%). Un-inoculated control roots were nearly half-infected (47.79% MkI), likely due to endogenous pathogens from the kernels, whereas un-inoculated roots treated with Strep_22 remained mostly healthy (10.87% MkI) (Panel c). Final re-isolations confirmed that Strep_22 protected roots against both artificially inoculated and endogenous pathogens. It significantly reduced re-isolation frequencies for *F. algeriense* (from 27.08% to 6.25%), *F. culmorum* (from 25.00% to 4.17%), and *F. oxysporum* f.sp. *dianthi* (from 22.92% to 2.08%). For other background fungi, isolation rates fell from 66.67%–100.00% in un-treated trials down to 6.25%–12.50% with Strep_22 (Panel d; Table S9). Specifically, the strain completely suppressed sterile mycelia and fungi from the genera *Aspergillus*, *Cladosporium*, *Epicoccum*, *Penicillium*, *Phoma*, *Stemphylium*, and *Trichothecium*, while significantly reducing *Alternaria* spp. and *Fusarium* spp. (Table S9).

Fig. 7 illustrates how root health affected leaf yellowing. Pathogens did not severely compromise the overall foliage, though yellowing was significantly higher in the older first leaf (23.99%–28.78% MkI) than in the younger second leaf (5.22%–5.67% MkI) (Panel a). Strep_22 root protection positively influenced the aerial parts (Panel b). Yellowing of the first (41.50% MkI) and second (10.47% MkI) leaves of un-treated plants was significantly higher than that observed in Strep_22-treated plants (10.80% and 0.47% MkI, respectively). Interestingly, Strep_22 provided greater protection to the second leaf than the first, reducing MkI values nearly 4-fold for the first leaf and almost 10-fold for the second. This disparity may stem from the different physiological ages of the leaves or indicate that Strep_22 efficacy diminishes over time. Panel c (Table S12) confirms that leaf yellowing is primarily driven by leaf age rather than pathogen infection. For both leaves, MkI values in un-treated, un-inoculated plants (43.63% and 10.72%, respectively) did not differ significantly from those in inoculated, un-treated plants (first leaf: 38.92%–43.85%; second leaf: 10.07%–10.47%). In contrast, significant differences were observed among the first leaves of Strep_22-treated plants. Mirroring the root results, *F. oxysporum* f.sp. *dianthi* (15.63% MkI) and *F. algeriense* (13.72% MkI) were less susceptible to the strain than *F. culmorum* (9.06% MkI). No significant differences occurred in the second leaves of treated plants, with MkI values ranging from 0.32% (Fox_d*Strep_22) to 0.86% (Fal*Strep_22). Regardless, Strep_22-treated plants consistently exhibited better leaf health than un-treated ones.

Fig. 8 and 9 illustrate how Strep_22 mitigated the inhibitory effects of fungal pathogens and promoted wheat growth. In the absence of Strep_22, un-treated plants (22.02 cm) were significantly taller than those infected by *F. oxysporum* f.sp. *dianthi* (20.03 cm), *F. algeriense* (19.80 cm), and *F. culmorum* (19.32 cm). In contrast, no significant differences were observed among the Strep_22-treated groups, which ranged from 39.43 cm (with *F. algeriense* and *F. oxysporum* f.sp. *dianthi*) to 40.56 cm (without *F. culmorum*). This indicates that the strain effectively neutralized the pathogens' negative impact on plant elongation and exerted a significant growth-promoting effect compared to un-treated controls (Panel a; Table s17). A similar trend was observed for dry matter bioaccumulation (Panel b). Without Strep_22, *F. culmorum* (22.55%), *F. oxysporum* f.sp. *dianthi* (21.72%), and *F. algeriense* (20.10%) significantly reduced biomass compared to un-treated plants (28.27%). Interestingly, Strep_22 mitigated rather than fully reversed this negative effect on dry matter. Plants treated with Strep_22 alone achieved higher bioaccumulation (37.36%) than those treated with the strain alongside *F. algeriense* (28.71%), *F. culmorum* (28.62%), or *F. oxysporum* f.sp. *dianthi* (28.34%). Nevertheless, across all identical fungal inocula, adding Strep_22 yielded significantly higher dry matter values compared to the un-treated trials.

3.3.3. Evaluation of the antifungal activity of strain Strep_22 against fungal phytopathogens in tomato

Consistent with the wheat experiment, Fig. 9 and 10 illustrate tomato root disease incidence, confirming the biocontrol efficacy of Strep_22. Tomato roots were highly susceptible to *A. rolfsii* (MkI = 29.17%), while *S. sclerotiorum* (14.93%) and *F. oxysporum* (13.89%) were less aggressive; un-treated control plants remained almost entirely healthy (MkI = 1.04%) (Panel a; Table S19). Strep_22 application provided significant root protection, reducing the overall MkI from 23.44% to 6.08% (Panel b). Treatment efficacy varied by pathogen (Panel c): Strep_22 significantly reduced root damage caused by *A. rolfsii* (from 43.06% to 15.29%) and *F. oxysporum* (from 27.08% to 0.69%), and to a lesser extent, *S. sclerotiorum* (from 22.92% to 6.94%). No significant differences occurred between un-inoculated trials with or without the strain (0.69% and 1.39%, respectively). Re-isolation assays confirmed that both inoculated and indigenous soil-borne fungi were sensitive to Strep_22 (Panel d; Table S22). Remarkably, none of the inoculated fungal pathogens were recovered from roots treated with Strep_22, whereas re-isolation frequencies in un-treated trials were 21.70% for *A. rolfsii*, 17.36% for *F. oxysporum*, and 13.02% for *S. sclerotiorum*. Strep_22 also suppressed background soil mycoflora. Without the strain, the isolation frequency of other fungi ranged from 33.30% to 64.58%, but dropped to between 8.33% and 12.50% in Strep_22-treated trials. Most of these background fungi were soil-borne, as they were absent in the initial seedlings. Among them, *Phoma* spp. were the most abundant in un-treated trials (20.83–41.67%) but were nearly eliminated by Strep_22 (2.08% across all trials).

Fig. 11 illustrates how soil treatments influenced the tomato plants' collars and aerial parts. Panel a indicates that pathogens impacted the aerial portions more severely than the collars: collar damage ranged from 2.08% (*S. sclerotiorum*) to 20.83% (*A. rolfsii*), while aerial damage spanned 23.96% (*S. sclerotiorum*) to 36.81% (*A. rolfsii*). While collar degradation is a direct consequence of fungal infection, aerial yellowing is an indirect result of root and collar decay, explaining the significantly higher damage values observed in the foliage. For both tissue types, *A. rolfsii* was the most aggressive pathogen (MkI = 20.83% collar, 36.81% aerial), followed by *F. oxysporum* (4.51% and 26.74%) and *S. sclerotiorum* (2.08% and 23.96%). No infection was recorded on the collars of un-treated control plants, though their aerial parts exhibited slight yellowing (MkI = 16.67%)

(Table S23). The protective effect of Strep_22 was consistent across both tissue types (Panel b). Plants treated with Strep_22 displayed lower MkI values in the collars (2.78%) and aerial parts (15.97%) compared to un-treated plants (10.94% and 36.11%, respectively). Panel c reveals the strain's efficacy against individual pathogens. Across all inoculated trials, Strep_22 significantly reduced the MkI of the collars (2.78%–8.33%) and aerial parts (14.58%–18.75%) compared to un-treated plants, where *A. rolfsii* damage reached up to 33.33% (collar) and 54.86% (aerial). Although not statistically significant, collar damage from *S. sclerotiorum* was slightly higher in Strep_22-treated plants than in un-treated controls. In un-inoculated trials, no collar damage was observed in either group, and aerial yellowing did not differ significantly between un-treated and Strep_22-treated plants (18.06% vs. 15.28%, respectively). The health status of the roots, collar, and aerial parts significantly influenced overall tomato plant growth (Fig. S4; Panels a–d). *A. rolfsii* infection most severely reduced final plant height (64.58 cm) compared to *S. sclerotiorum* (69.66 cm), *F. oxysporum* (70.65 cm), and the un-treated control (85.39 cm) (Panel a; Table S26). However, the general protection provided by Strep_22 resulted in significantly taller plants than those in un-treated trials, reaching 80.31 cm and 64.83 cm, respectively (Panel b; Table S27). Biomass accumulation followed a identical trend (Panels c, d). *A. rolfsii* was the most aggressive pathogen, reducing dry matter to 13.81%, followed by *F. oxysporum* (16.15%) and *S. sclerotiorum* (16.60%), compared to un-treated controls (23.85%) (Panel c; Table S28). Accordingly, Strep_22-treated plants achieved significantly higher bioaccumulation than un-treated ones, with dry matter values of 20.10% and 15.18%, respectively (Panel d; Table S29).

Fig. 12 illustrates the interaction between Strep_22 and the pathogens on tomato growth parameters. Regarding final plant height, Strep_22 neutralized the inhibitory effects of the pathogens but did not exert an independent growth-promoting effect (Panel a). Within trials inoculated with the same fungi, Strep_22-treated plants, measuring 75.04 cm (*A. rolfsii*), 80.00 cm (*S. sclerotiorum*), and 80.35 cm (*F. oxysporum*), were significantly taller than their un-treated counterparts (54.13 cm, 59.31 cm, and 60.94 cm, respectively). However, these values did not differ significantly from the un-inoculated, un-treated control (84.94 cm). Because no significant difference was observed between the un-treated control and the Strep_22-only trial (85.83 cm), the strain did not inherently promote plant elongation (Panel a; Table S30). Regarding biomass accumulation, only *A. rolfsii* significantly reduced dry matter (10.67%) compared to un-treated plants (17.11%), whereas *S. sclerotiorum* (16.19%) and *F. oxysporum* (16.56%) had no significant impact (Panel b; Table S31). In these interactions, Strep_22 statistically increased dry matter only in the *A. rolfsii* trial (16.95%), showing no significant increase in plants inoculated with *F. oxysporum* (15.73%) or *S. sclerotiorum* (17.02%). However, in contrast to the plant height results, un-inoculated plants treated with Strep_22 alone achieved significantly higher dry matter (30.58%) than the un-treated controls, demonstrating an inherent growth-promoting effect on biomass.

4 DISCUSSION

Modern agriculture faces major challenges as strict EU regulations limit or ban synthetic pesticides due to their risks to human health and the environment, making the registration of new chemicals long and expensive.[44-46] Consequently, the sector is shifting toward sustainable alternatives like plant-derived products and microbial Biocontrol Agents (BCAs). Recent studies highlight the efficacy of botanical fungicides, particularly from *Eucalyptus camaldulensis*, *Citrus bergamia*, *Solanum lycopersicum*, and *Punica granatum*, in suppressing over 30 phytopathogens and limiting disease severity.[37,47] Similarly, microbial BCAs like *Trichoderma* spp. and *Bacillus* spp. effectively control broad-spectrum fungal pathogens such as *Alternaria alternata*, *Aspergillus nidulans*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Penicillium crustosum*.[48] These BCAs operate through mechanisms like cell-wall degrading enzymes (chitinase and β-1,3-glucanase) from *Trichoderma* spp., and IAA production or phosphate solubilization from *Bacillus* spp., which successfully inhibit pathogens like *Bipolaris sorokiniana in vitro* and *in vivo*, while their integration with *Aloe vera* further improves wheat growth parameters.[48,49] Recently, research has focused on *Streptomyces* spp. as potential BCAs for sustainable pathogen management. These Gram-positive soil bacteria grow via hyphal tip extension and branching to form a substrate mycelium that interacts with plant root hairs, providing biological benefits. In response to environmental stress, they transition from a vegetative phase to a reproductive sporulation phase, forming an aerial mycelium.[50-52] During this morphological differentiation, their genome utilizes biosynthetic gene clusters (BGCs) to produce secondary metabolites, such as polyketides, lactams, non-ribosomal peptides, and terpenes, with potent antimicrobial, growth-promoting, and herbicidal activities.[53,54] Owing to these traits, *Streptomyces* spp. exhibit high efficacy against a broad range of devastating fungal pathogens.[24,55] Based on

these characteristics, a specific strain designated Strep_22 (selected alongside Strep_23 and Strep_24) demonstrated the superior *in vitro* inhibitory activity against ten major soilborne fungi (including *Agroathelia rolfsii*, *Sclerotinia sclerotiorum*, and various *Fusarium* spp.) that severely impact wheat and tomato crops in the Foggia province. Prior to greenhouse trials, mycological isolations revealed that nearly all selected wheat kernels were naturally infested with endophytic fungal pathogens, whereas tomato roots showed high baseline health, with *Alternaria* spp., *Fusarium* spp., and sterile mycelium being the most abundant native microbes in both tissues. *In vivo* experiments confirmed that the Strep_22 strain is an excellent biocontrol agent (BCA). It successfully limited infections by *F. culmorum* and *F. oxysporum* f.sp. *dianthi* in wheat, and significantly reduced *A. rolfsii*, *F. oxysporum*, and *S. sclerotiorum* damage in tomato. Overall, Strep_22 emerged as the most effective strain, followed closely by Strep_24, with both displaying high inhibitory activity (IA) across all tested pathogens, whereas Strep_23 showed narrow-spectrum IA. Among the pathogens, *V. dahliae* and *F. avenaceum* were the most sensitive to all three strains.[56,57] Previous studies support these findings: *S. albidoflavus* suppresses *V. dahliae in vitro* and *in vivo*, partly because *Streptomyces*-produced albocycline inhibits conidiospore germination.[23,58] Furthermore, chitinolytic activity by *Streptomyces* strains drives major biocontrol efficacy, often exceeding 50% to 70%, against *Fusarium* diseases.[59] While Strep_22 was the top performer overall, it exhibited lower IA against *R. solani* and *V. dahliae* than Strep_24. However, Strep_22 completely inhibited *A. rolfsii*, and both strains achieved total inhibition of *S. sclerotiorum*. This strong performance aligns with literature showing that *Streptomyces* strains disrupt the hyphae and organelles of *R. solani* and degrade the cell walls of *A. rolfsii* via extracellular enzymes.[60,61]

In the wheat experiment, neither *Fusarium* spp. infections nor Strep_22 treatment affected kernel germination, aligning with findings that *Fusarium* does not significantly alter maize germination rates.[62] Among the pathogens, *F. algeriense* and *F. oxysporum* f.sp. *dianthi* were the most pathogenic to wheat roots. This is supported by studies identifying *F. oxysporum* as a predominant isolate from infected roots/crowns and *F. algeriense* as a cause of moderate crown rot in wheat.[63,64]

Strep_22 significantly reduced root infections across all pathogens, showing exceptional efficacy against *F. culmorum* and *F. oxysporum* f.sp. *dianthi*. This mirrors previous research where *Streptomyces* strains outperformed chemical fungicides in inhibiting *F. oxysporum* root infections.[65] Fungal re-isolations confirmed that Strep_22 significantly lowered the frequency of artificially inoculated pathogens and successfully controlled endogenous microbiota originating from the kernels. Mechanistically, *Streptomyces* secondary metabolites, such as salvianolic acid B, are known to cause spore lysis, hyphal distortion, and cellular leakage in pathogens like *A. brassicicola* while enhancing plant agronomic traits.[66] Furthermore, volatile or diffusible secondary metabolites from other *Streptomyces* strains have demonstrated distance-based mycelial and conidial germination inhibition against fungi like *P. verrucosum*, *A. fumigatus*, and *F. verticillioides*.[67]

In the tomato experiment, un-inoculated trials showed no baseline root infection, confirming the excellent initial health of the seedlings. Among the introduced pathogens, *A. rolfsii* proved to be the most aggressive and destructive on tomato roots.[68] Nevertheless, treatment with Strep_22 significantly reduced disease incidence across all inoculated pathogens, mirroring the biocontrol efficacy observed in the wheat experiment. This robust protection aligns with recent literature; for instance, spore powder of *S. flavotricini* SF25 significantly reduces *A. rolfsii* southern blight severity, inhibits sclerotia formation, promotes beneficial soil microbes, and upregulates systemic acquired resistance (SAR) genes to yield healthier leaves.[69] Similarly, *Streptomyces* culture filtrates are known to suppress *F. oxysporum* on tomato seedlings via direct mycelial growth inhibition.[70] Re-isolation assays from tomato roots verified that Strep_22 successfully suppressed the inoculated pathogens as well as background soil fungi, including *Alternaria* spp., *Aspergillus* spp., and *Fusarium* spp. A parallel trend was recorded for the collar and aerial parts: *A. rolfsii* emerged as the most damaging pathogen to these tissues, but Strep_22 significantly mitigated the damage across all pathogen groups.

Finally, artificial inoculation negatively impacted tomato growth parameters, with *A. rolfsii* causing the sharpest declines in height and dry matter, a suppressive effect heavily tied to host genotype resistance (Ahmed and Shoaib, 2025). While the growth-promoting effect was less pronounced here than in the wheat trials, Strep_22 significantly enhanced both plant height and biomass accumulation. This validates findings by[70] demonstrating that *Streptomyces* applications actively stimulate tomato growth, resulting in significantly increased shoot elongation, root elongation, and overall plant weight compared to untreated controls.

5 Conclusions

This study highlights the strain Strep_22 as a promising biocontrol agent (BCA) and plant growth promoter (PGP) for wheat and tomato crops. *In vitro* trials demonstrated its superior, near-total inhibitory capacity against various soilborne fungal pathogens, particularly *Agroathelia rolfsii*, *Fusarium solani*, and *Sclerotinia sclerotiorum*. This efficacy was confirmed in greenhouse experiments, where Strep_22 drastically reduced

root and collar disease incidence (MkI), protecting plants from both artificial inocula and endogenous kernel microbiota. Beyond crop protection, the strain neutralized pathogen-induced growth suppression, significantly stimulating tissue elongation and dry matter accumulation. This dual role as a protector and biostimulant is linked to bioactive secondary metabolite production and enhanced physiological health, which minimized systemic symptoms like leaf yellowing. In a regulatory context strictly limiting synthetic pesticides, Strep_22 offers a sustainable, high-performance solution for Mediterranean disease management, ensuring yield stability and environmental integrity. However, whole-genome sequencing is required to resolve its precise taxonomic identity, as BLAST alignments proved inadequate by suggesting multiple distinct species. Concurrently, *in vitro* bioassays are underway to characterize the bioactive metabolites, evaluate their potential phytotoxicity, and establish efficacy thresholds. Ongoing studies aim to identify, quantify, and track the specific metabolites driving plant protection and bioaccumulation.

CRediT authorship contribution statement

Thomas Conte and Antonia Carlucci: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Maria Grazia Morea, Gaetana Ricciardi and Angelo Pastino**: Methodology and investigation. **Francesco Lops** and **Maria Luisa Raimondo**: Writing – review & editing. **Antonia Carlucci**: Supervision, Funding acquisition and Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflicts of Interest

The authors declare no conflicts of interest.

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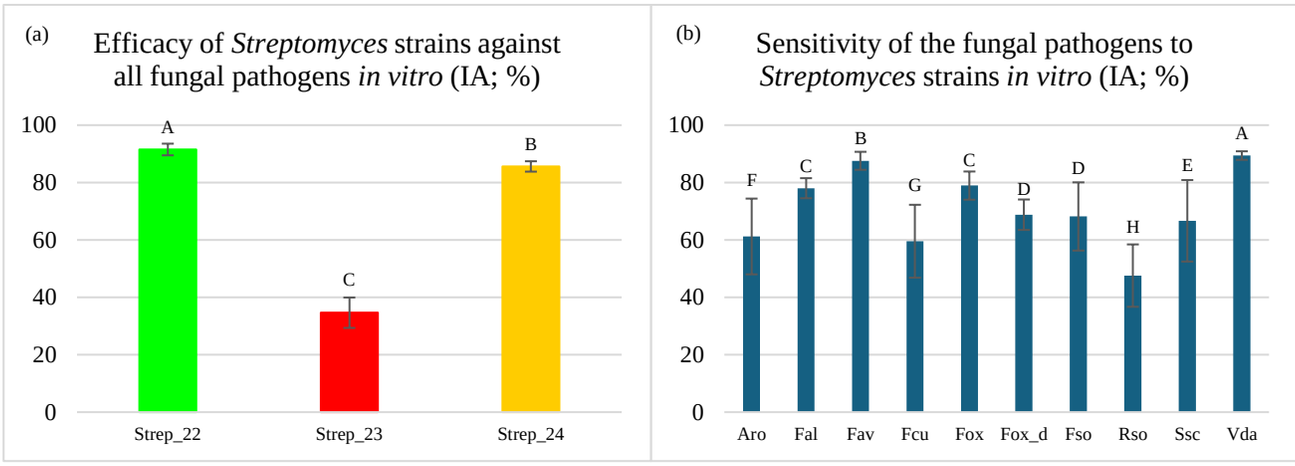


Figure 1. Inhibitory Activity (IA; %) of *Streptomyces* strains under *in vitro* conditions: (a) inhibitory activity played by three different *Streptomyces* strains against all tested fungal pathogens; (b) sensitivity of the fungal pathogens to the presence of the *Streptomyces* strains Aro: *Agroathelia rolfsii*; Fal: *Fusarium algeriense*; Fav: *F. avenaceum*; Fcu: *F. culmorum*; Fox: *F. oxysporum*; Fox_d: *F. oxysporum* f.sp. *dianthi*; Fso: *F. solani*; Rso; *Rhizoctonia solani*; Ssc: *Sclerotinia sclerotiorum*; Vda: *Verticillium dahliae*. One-way Anova, Tukey Test ($p < 0.01$).

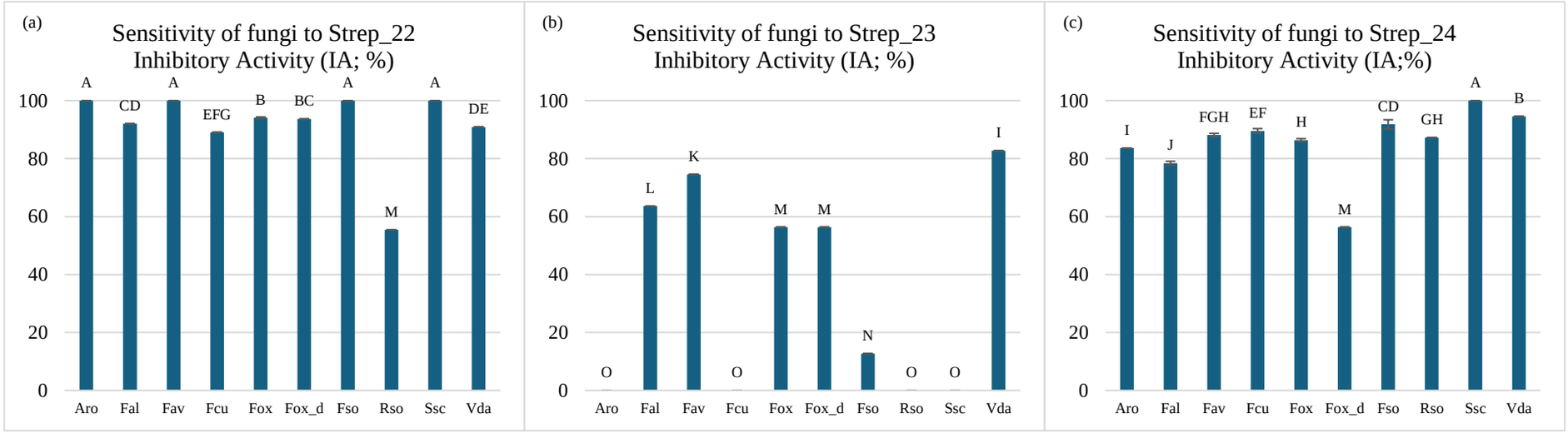


Figure 2. Efficacy of the individual strains in controlling fungal pathogens expressed as inhibitory activity (IA; %). Sensitivity of fungal pathogens to *Streptomyces* strains: Strep_22 (a); Strep_23 (b); and Strep_24 (c). Aro: *Agroathelia rolfsii*; Fal: *Fusarium algeriense*; Fav: *F. avenaceum*; Fcu: *F. culmorum*; Fox: *F. oxysporum*; Fox_d: *F. oxysporum* f.sp. *dianthi*; Fso: *F. solani*; Rso; *Rhizoctonia solani*; Ssc: *Sclerotinia sclerotiorum*; Vda: *Verticillium dahliae*. Data were analysed using Factorial ANOVA followed by Tukey's test ($p< 0.01$).

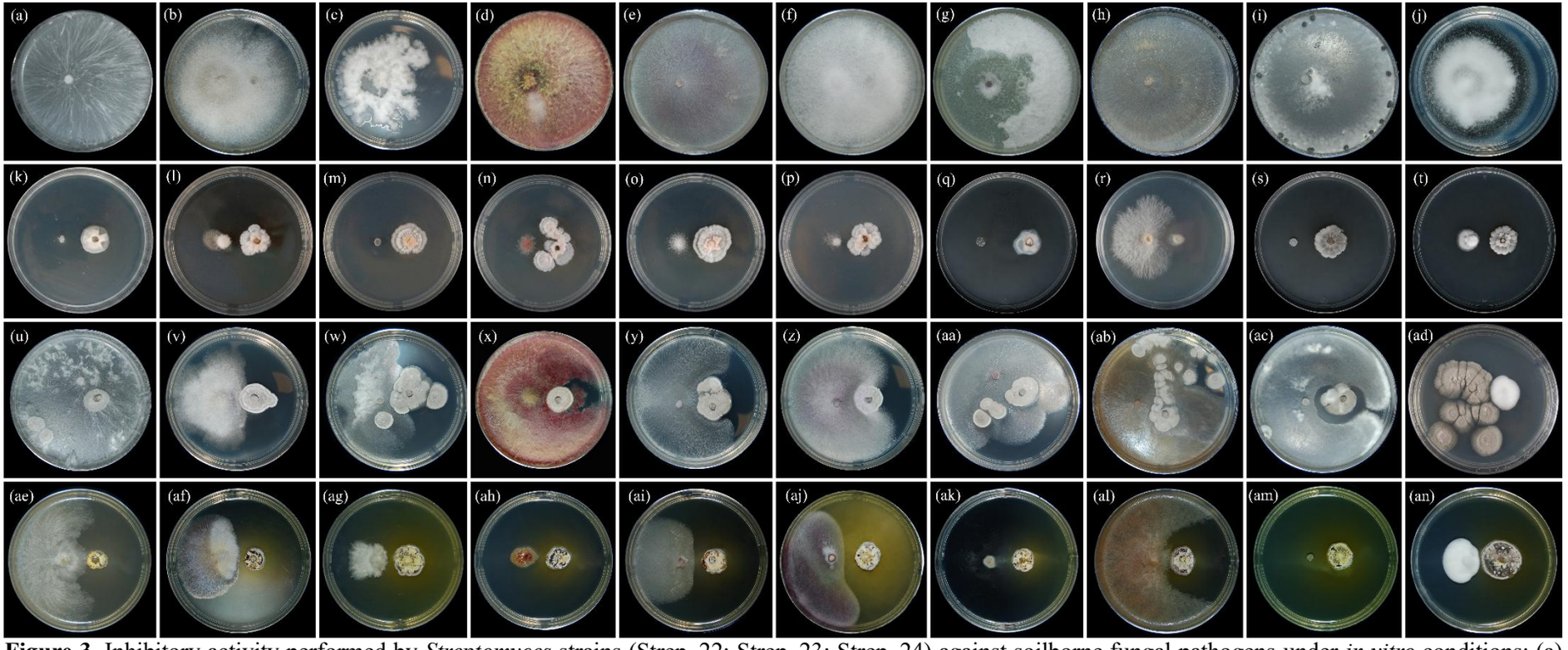


Figure 3. Inhibitory activity performed by *Streptomyces* strains (Strep_22; Strep_23; Strep_24) against soilborne fungal pathogens under *in vitro* conditions: (a) *Agroathelia rolfsii* (Aro) control plate; (b) *Fusarium algeriense* (Fal) control plate; (c) *F. avenaceum* (Fav) control plate; (d) *F. culmorum* (Fcu) control plate; (e) *F. oxysporum* (Fox) control plate; (f) *F. oxysporum* f.sp. *dianthi* (Fox_d) control plate; (g) *F. solani* (Fso) control plate; (h) *Rhizoctonia solani* (Rso) control plate; (i) *Sclerotinia sclerotiorum* (Ssc) control plate; (j) *Verticillium dahliae* (Vda) control plate; (k) Aro*Strep_22; (l) Fal*Strep_22; (m) Fav*Strep_22; (n) Fcu*Strep_22; (o) Fox*Strep_22; (p) Fox_d*Strep_22; (q) Fso*Strep_22; (r) Rso*Strep_22; (s) Ssc*Strep_22; (t) Vda*Strep_22; (u) Aro*Strep_23; (v) Fal*Strep_23; (w) Fav*Strep_23; (x) Fcu*Strep_23; (y) Fox*Strep_23; (z) Fox_d*Strep_23; (aa) Fso*Strep_23; (ab) Rso*Strep_23; (ac) Ssc*Strep_23; (ad) Vda*Strep_23; (ae) Aro*Strep_24; (af) Fal*Strep_24; (ag) Fav*Strep_24; (ah) Fcu*Strep_24; (ai) Fox*Strep_24; (aj) Fox_d*Strep_24; (ak) Fso*Strep_24; (al) Rso*Strep_24; (am) Ssc*Strep_24; (an) Vda*Strep_24.

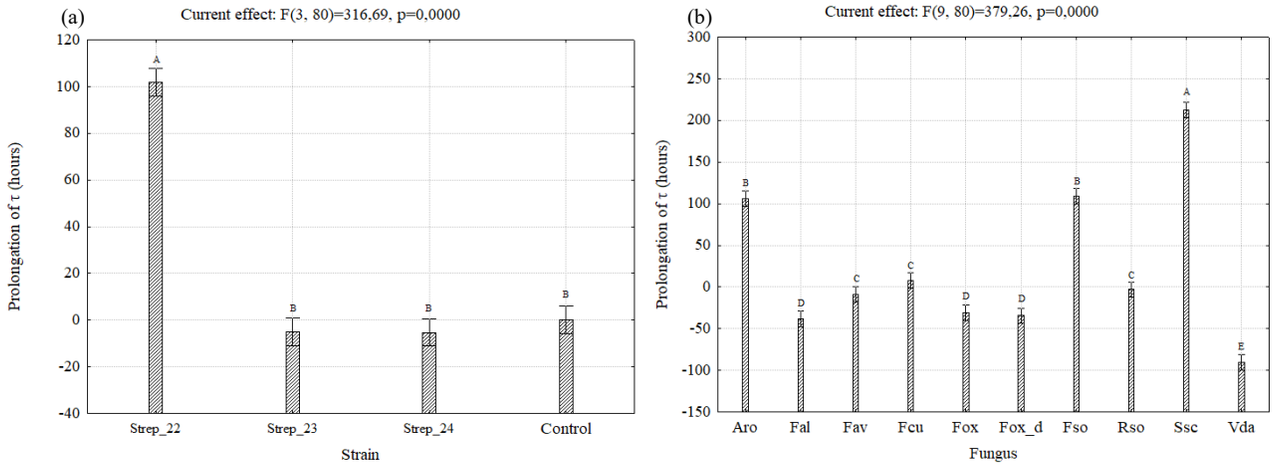


Figure 4. Inhibition of fungal pathogen growth by *Streptomyces* strains expressed as Δτ: (a) effect of individual strain on the growth rate of fungal pathogens; (b) changes in the growth rate of fungal pathogens in dual culture compared to the control. Aro: *Agroathelia rolfsii*; Fal: *Fusarium algeriense*; Fav: *F. avenaceum*; Fcu: *F. culmorum*; Fox: *F. oxysporum*; Fox_d: *F. oxysporum* f.sp. *dianthi*; Fso: *F. solani*; Rso; *Rhizoctonia solani*; Ssc: *Sclerotinia sclerotiorum*; Vda: *Verticillium dahliae*. Planned comparisons (hypothesis decomposition) of the Factorial ANOVA highlight the significant effect of the *Streptomyces* strain type on inhibitory activity.

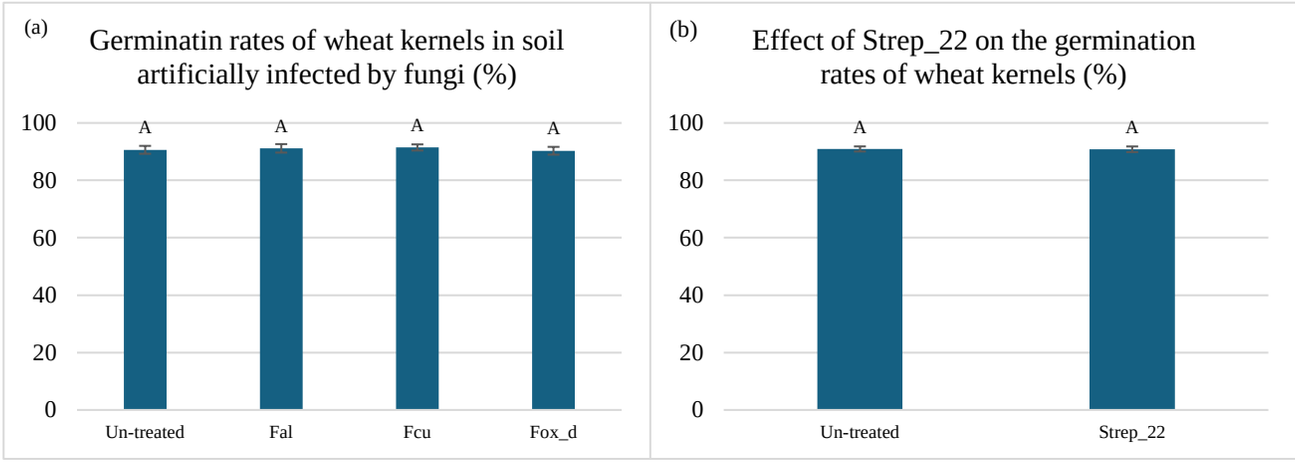


Figure 5. Germination of wheat kernels under the *in vivo* wheat experiment: (a) influence of inoculated fungal pathogens on kernel germination; (b) effect of strain Strep_22 on kernel germination. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. One-way ANOVA followed by Tukey's test ($p < 0.01$) was performed; means followed by the same uppercase letter are not significantly different.

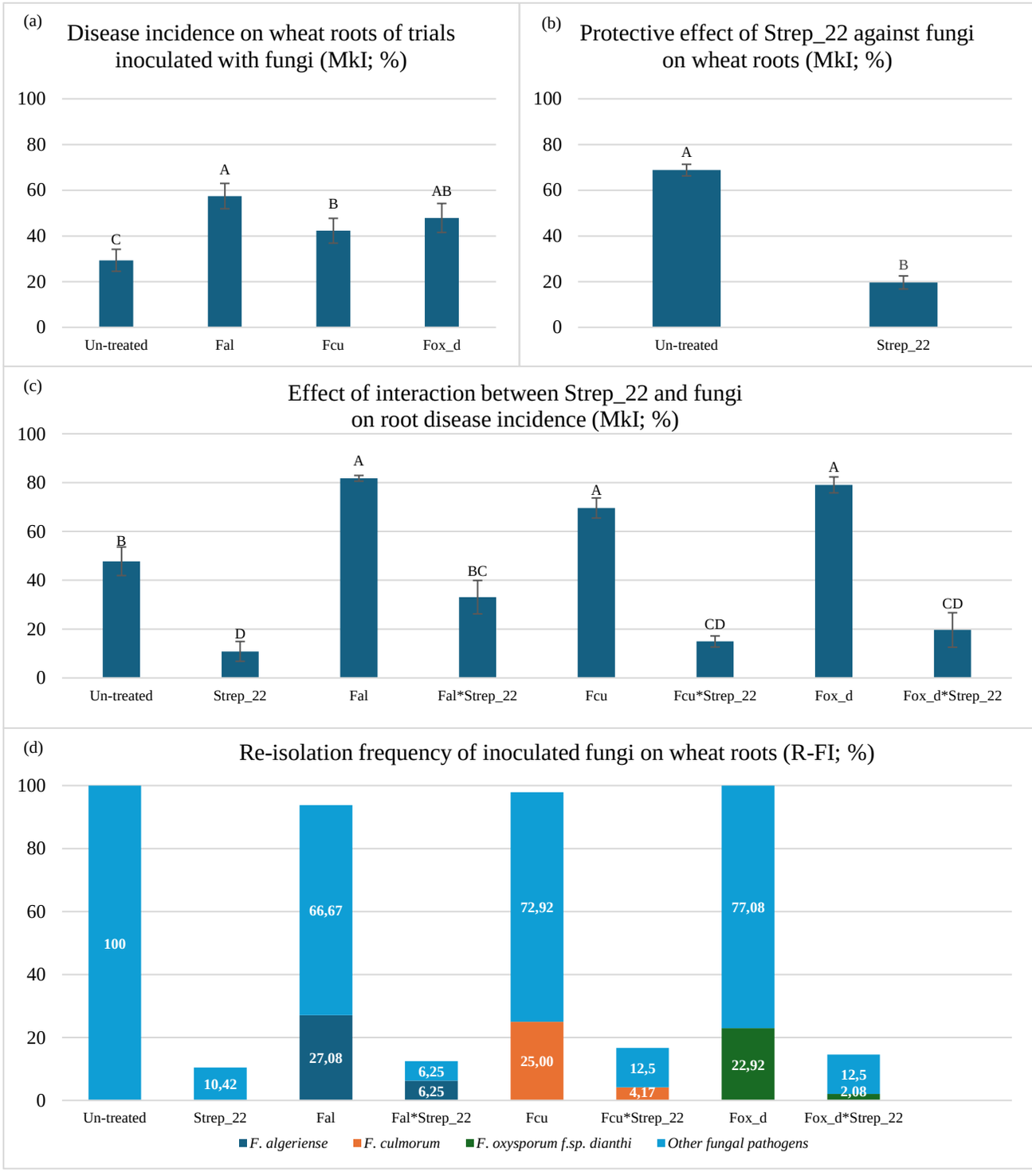


Figure 6. Damage observed on wheat roots following inoculation with fungal pathogens: (a) disease incidence on wheat roots inoculated with fungal pathogens in presence of Strep_22; (b) biocontrol efficacy (protection) of strain Strep_22 against the fungal pathogens; (c) interaction between Strep_22 and individual fungal pathogens expressed as root disease incidence (MkI; %) of the roots; (d) re-isolation percentage of the inoculated fungal pathogens from the roots at the end of the experiment. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. One-way and Factorial ANOVA (interaction: Strep_22 vs fungal pathogens) followed by Tukey's test ($p< 0.01$) were performed; means followed by the same uppercase letter are not significantly different.

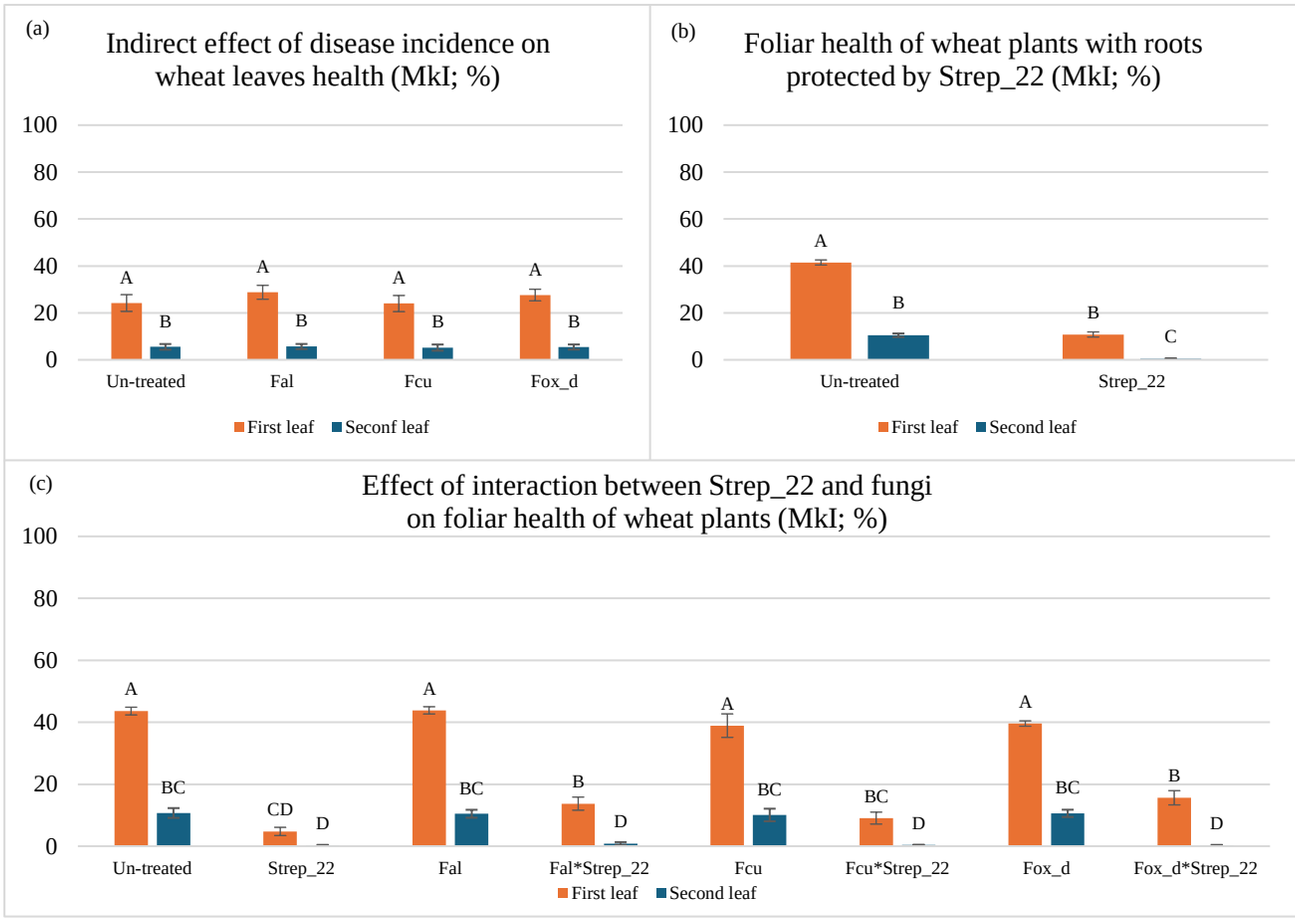


Figure 7. Disease status of wheat plants by leaf yellowing due to root damage by artificial fungal inoculation: (a) yellowing of the first and second leaves caused by individual fungal pathogens; (b) effect of root protection by Strep_22 on the phytosanitary status of the first and second leaves; (c) comparison of leaf yellowing between un-treated trials and those treated with Strep_22. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Data were analysed using One-way and Factorial ANOVA (interaction: Strep_22 × fungal pathogens) followed by Tukey's Test ($p < 0.01$). Means sharing the same capital letter are not significantly different.

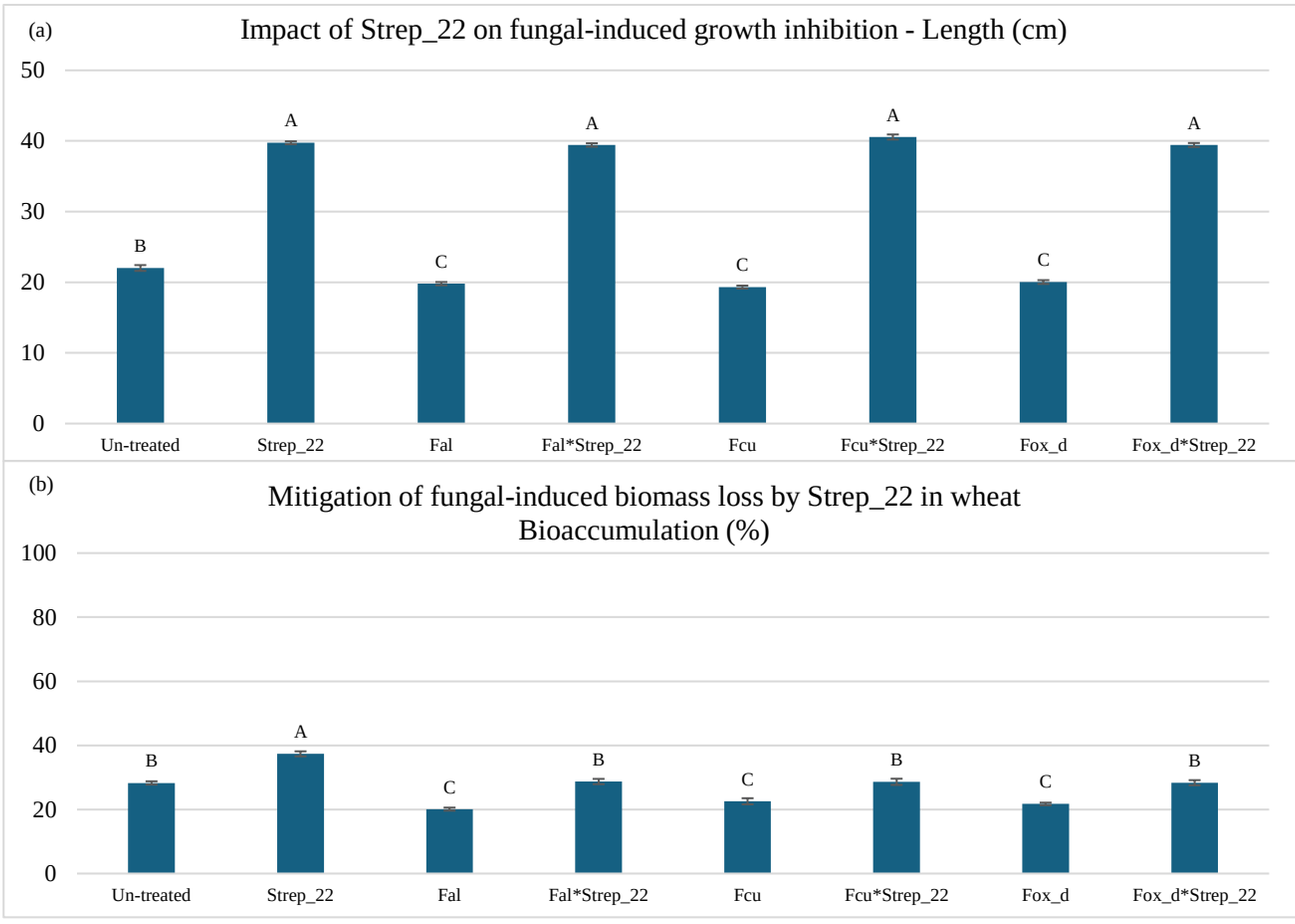


Figure 8. Effect of the interaction between Strep_22 and fungal pathogens on the growth parameters of wheat plants: (a) mitigation of pathogen-induced growth inhibition and promotion of plant length by Strep_22; (b) mitigation of pathogen-induced biomass reduction and promotion of bioaccumulation by Strep_22. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Data were analysed via Factorial ANOVA (interaction: Strep_22 × fungal pathogens) followed by Tukey's test ($p < 0.01$). Means sharing the same capital letter are not significantly different.

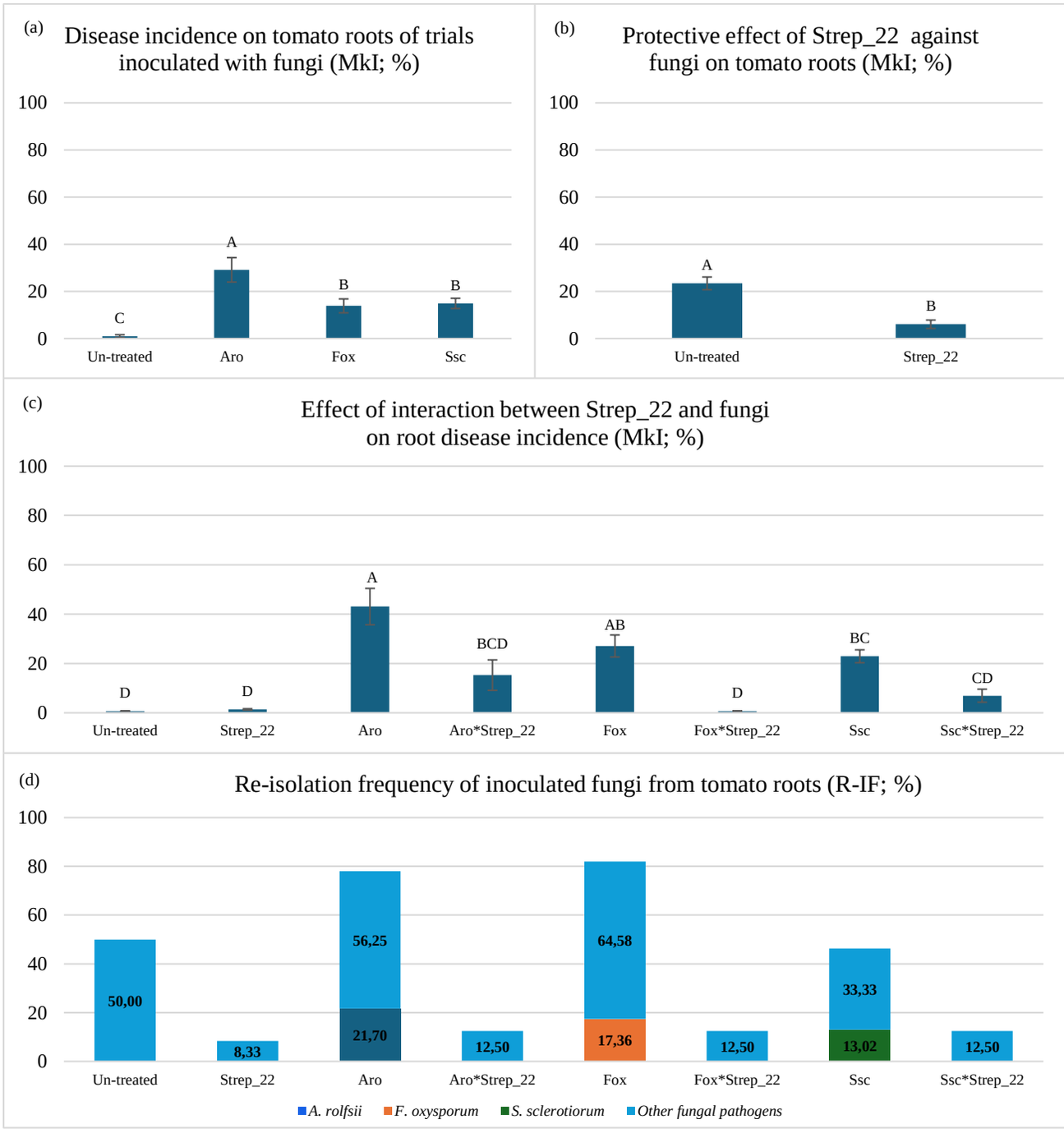


Figure 9. Assessment of fungal pathogen damage and the biocontrol efficacy of Strep_22 on tomato roots: (a) disease incidence (%) of inoculated fungal pathogens on tomato roots; (b) protective effect of strain Strep_22 against fungal pathogens on the root system; (c) interaction between Strep_22 and individual fungal pathogens expressed as Disease Incidence (MkI; %) of the roots; and (d) re-isolation frequency (%) of the inoculated fungal pathogens from tomato roots at the end of the experiment. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. One-way and Factorial ANOVA (interaction: Strep_22 vs fungal pathogens) followed by Tukey's test ($p< 0.01$) were performed; means followed by the same uppercase letter are not significantly different.



Figure 10. Phytosanitary status of un-treated and treated wheat and tomato plants after the greenhouse experiment: (a) un-treated wheat; (b) wheat plants treated with Strep_22; (c) un-treated tomato; (d) tomato plants treated with Strep_22; (e) un-treated wheat roots phytosanitary status; (f) *Fusarium algeriense* (Fal) infection on wheat roots; (g) *F. culmorum* (Fcu) infection on wheat roots; (h) *F. oxysporum* f.sp. *dianthi* (Fox_d) infection on wheat roots; (i) un-treated tomato roots phytosanitary status; (j) *Agroathelia rolfsii* (Aro) infection on tomato roots; (k) *F. oxysporum* (Fox) infection on tomato roots; (l) *Sclerotinia sclerotiorum* (Ssc) infection on tomato roots; (m) phytosanitary status of wheat roots protected by Strep_22; (n) Strep_22 protection against *F. algeriense* (Fal*Strep_22) infection on wheat roots; (o) Strep_22 protection against *F. culmorum* (Fcu*Strep_22) infection on wheat roots; (p) Strep_22 protection against *F. oxysporum* f.sp. *dianthi* (Fox_d*Strep_22) infection on wheat roots; (q) phytosanitary status of tomato roots protected by Strep_22; (r) Strep_22 protection against *A. rolfsii* (Aro*Strep_22) infection on tomato roots; (s) Strep_22 protection against *F. oxysporum* (Fox*Strep_22) infection on tomato roots; (t) Strep_22 protection against *Sclerotinia sclerotiorum* (Ssc*Strep_22) infection on tomato roots.

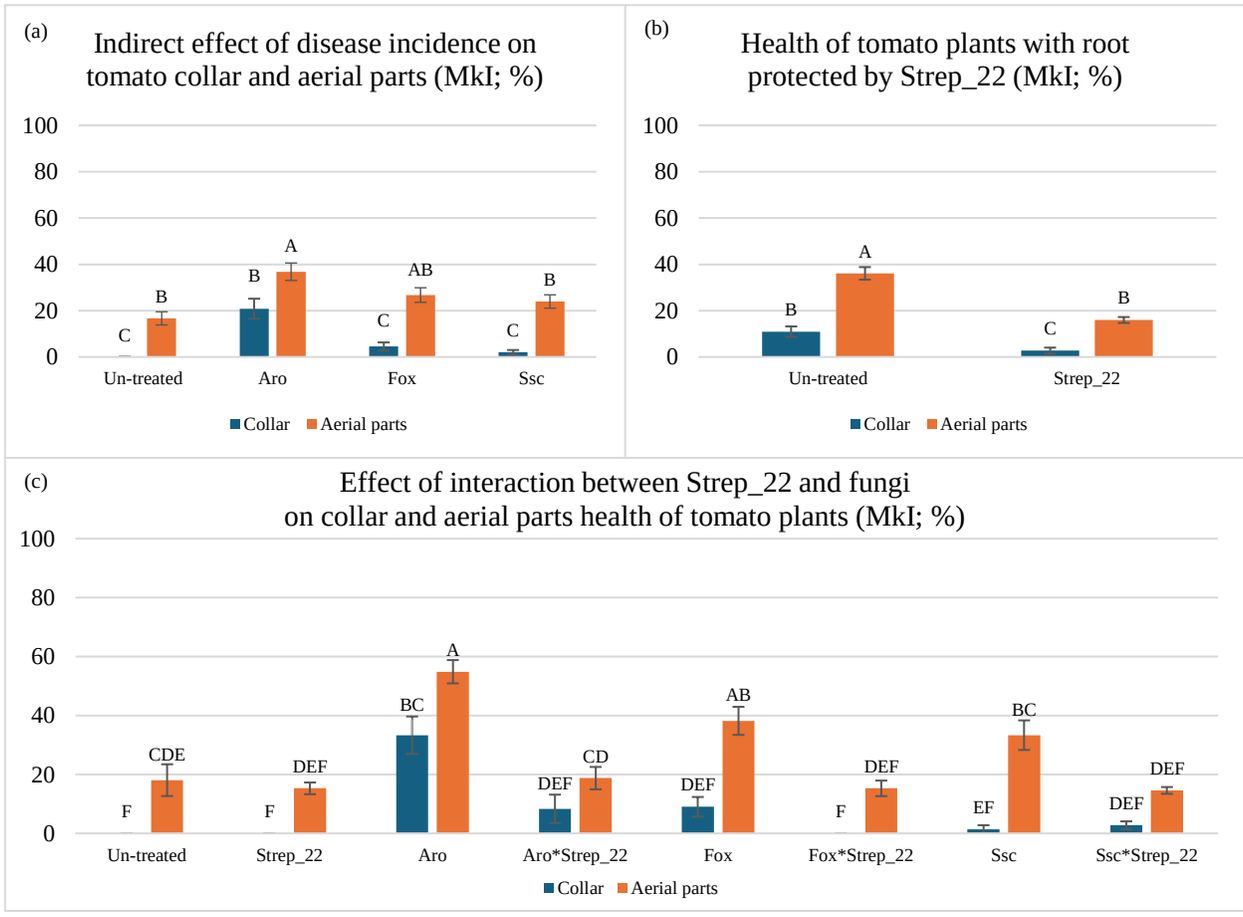


Figure 11. Phytosanitary status of the tomato plant collar and aerial parts: (a) damage to the collar and aerial tissues caused by individual fungal pathogens; (b) protective effect of Strep_22 on the health of the collar and aerial parts; and (c) comparative analysis of the phytosanitary status of the collar and aerial parts between untreated and Strep_22-treated trials. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. One-way and Factorial ANOVA (interaction: Strep_22 × fungal pathogens) followed by Tukey's Test ($p < 0.01$). Means sharing the same capital letter are not significantly different.

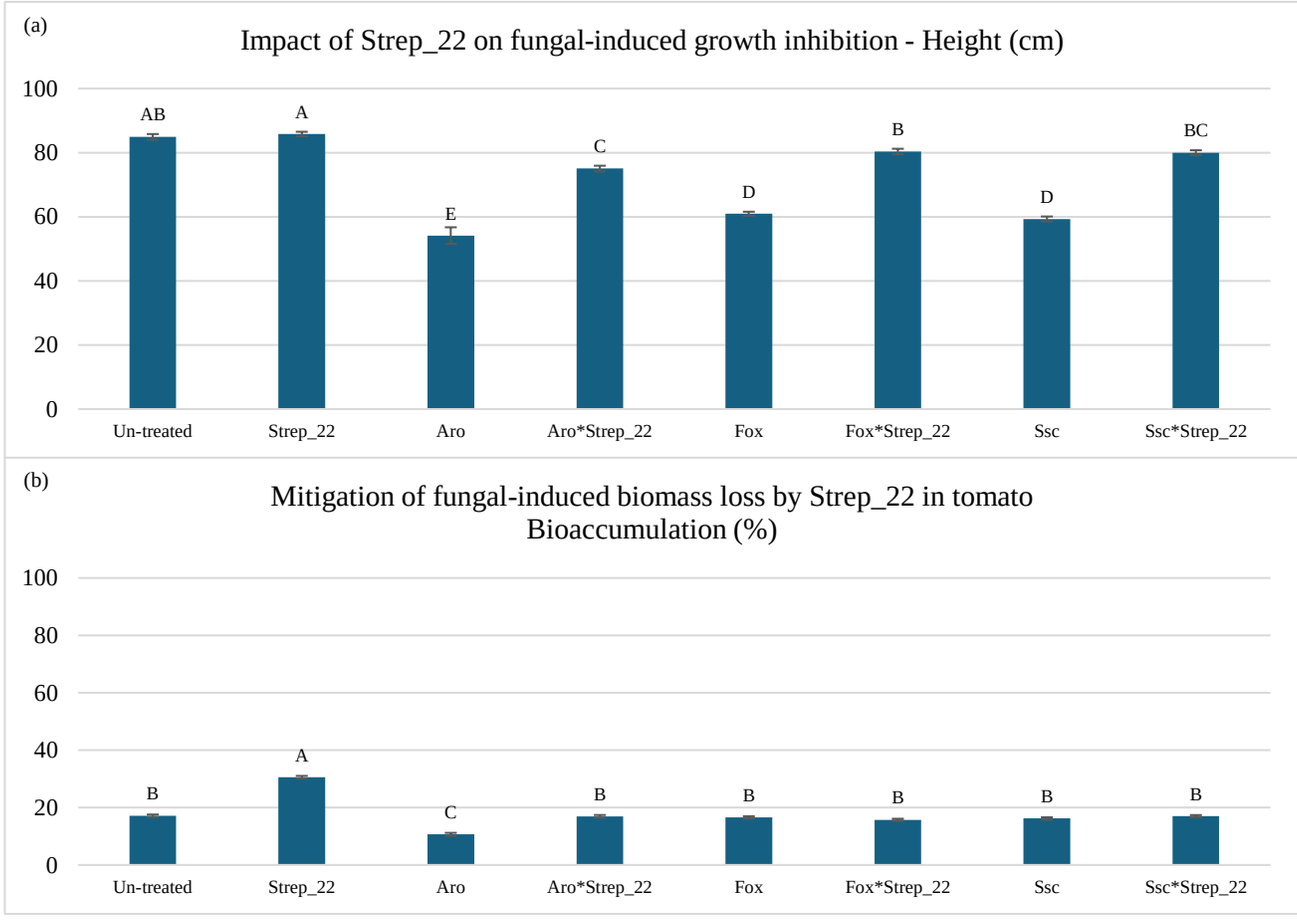


Figure 12. Effect of the interaction between Strep_22 and fungal pathogens on the growth parameters of tomato plants: (a) mitigation of pathogen-induced growth inhibition and promotion of plant height by Strep_22; (b) mitigation of pathogen-induced biomass reduction and promotion of bioaccumulation by Strep_22. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. Data were analysed via Factorial ANOVA (interaction: Strep_22 × fungal pathogens) followed by Tukey's test ($p < 0.01$). Means sharing the same capital letter are not significantly different.

Table 1. List of trials of treatments carried out in the *in vivo* wheat and tomato experiments.

Wheat experiment		
Treatment	**Fungal pathogen**	**Antagonist**
Un-treated	-[§]	-
Strep_22	-	Strep_22
Fal	*Fusarium algeriense*	-
Fal*Strep_22	*F. algeriensis*	Strep_22
Fcu	*F. culmorum*	-
Fcu*Strep_22	*F. culmorum*	Strep_22
Fox_d	*F. oxysporum* f.sp. *dianthi*	-
Fox_d*Strep_22	*F. oxysporum* f.sp. *dianthi*	Strep_22
Tomato experiment		
Un-treated	-	-
Strep_22	-	Strep_22
Aro	*Agroathelia rolfsii*	-
Aro*Strep_22	*A. rolfsii*	Strep_22
Fox	*Fusarium oxysporum*	-
Fox*Strep_22	*F. oxysporum*	Strep_22
Ssc	*Sclerotinia sclerotiorum*	-
Ssc*Strep_22	*S. sclerotiorum*	Strep_22

[§] No treatment.

Table 2. Fungal pathogens isolated from wheat kernels and roots of tomato seedlings used before performing the *in vivo* experiments.

Wheat kernels		**Tomato roots**	
Fungal pathogen	**N° infected kernels**	**Fungal pathogen**	**N° infected root tissues**
Alternaria spp.	23 (23.00%)	*Alternaria* spp.	3 (3.00 %)
Aspergillus spp.	11 (11.00%)	*Aspergillus* spp.	1 (1.00%)
Fusarium spp.	27 (27.00%)	*Fusarium* spp.	5 (5.00%)
Penicillium spp.	7 (7.00%)	*Penicillium* spp.	1(1.00%)
Stemphylium spp.	7 (7.00%)	*Stemphylium* spp.	1 (1.00%)
Sterile mycelium	15 (15.00%)	Sterile mycelium	6 (6.00%)
Bacteria	4 (4.00%)	Bacteria	7 (7.00%)
Subtotal microbial infections on wheat kernels	*94 (94.00%)*	*Subtotal microbial infections on tomato root tissue*	*24 (24.00%)*
No growth	6 (6.00%)	No growth	76 (71.00%)
Total	*100 (100.00%)*	*Total*	*100 (100.00%)*

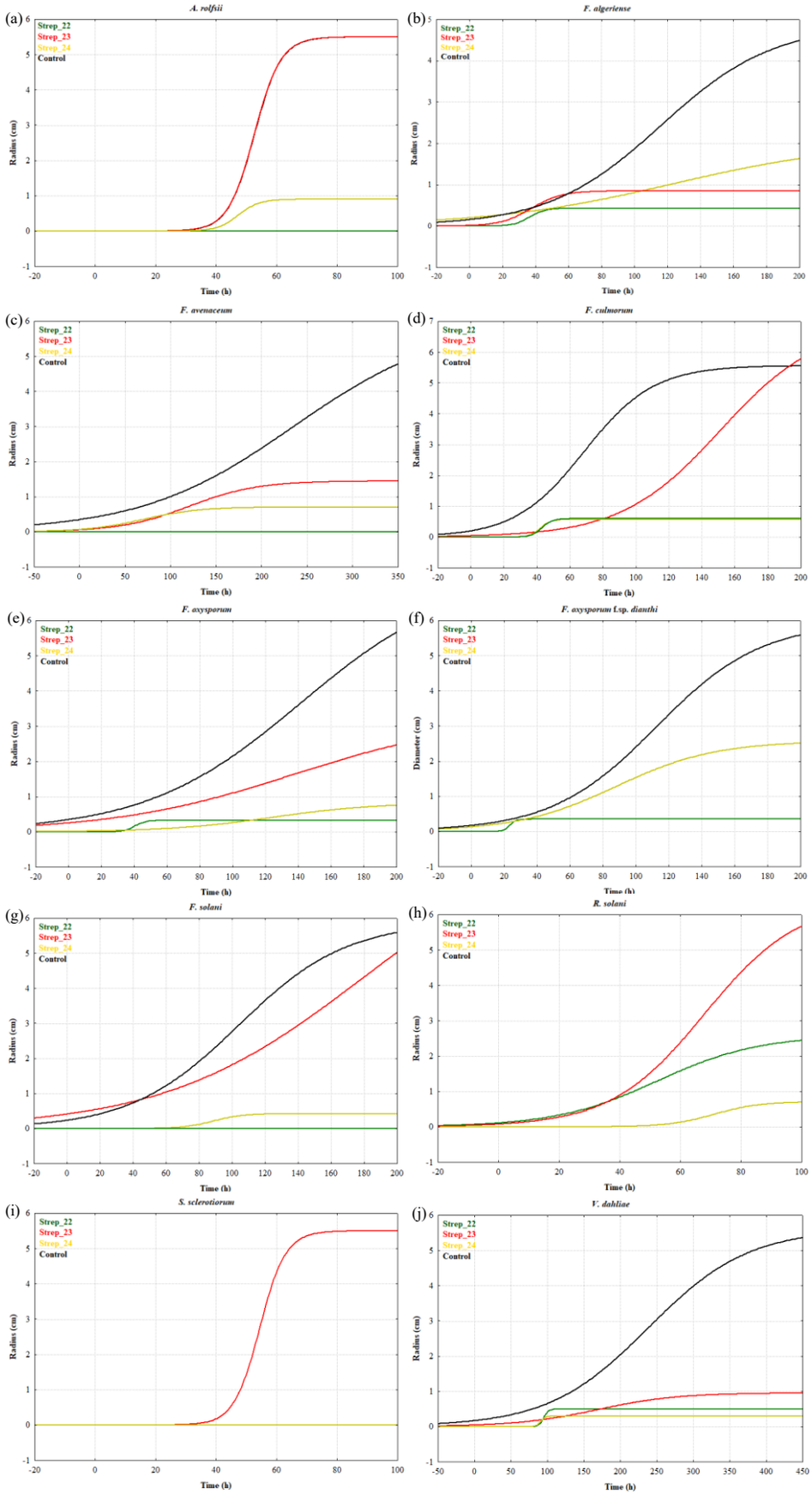


Figure S1. Growth Kinetics of tested fungal pathogens in dual culture with the *Streptomyces* strains: (a) *Agroathelia rolfsii* growth, Strep_23 line overlaps control line; (b) *Fusarium algeriensis* growth; (c) *F. avenaceum* growth; (d) *F. culmorum* growth; (e) *Fusarium oxysporum* growth; (f) *F. oxysporum* f.sp. *dianthi* growth, control line overlaps Strep_23 line; (g) *F. solani* growth; (h) *Rhizoctonia solani* growth, Strep_23 line overlaps control line; (i) *Sclerotinia sclerotiorum* growth, Strep_23 line overlaps control line, Strep_24 line overlaps Strep_22 line; (j) *Verticillium dahliae* growth. Fungal growth in dual culture with sterile medium plug was used as control. The coloured lines represent the fungal kinetics as the best fit of the data elaborated by the model of Dantigny et al. (2011).

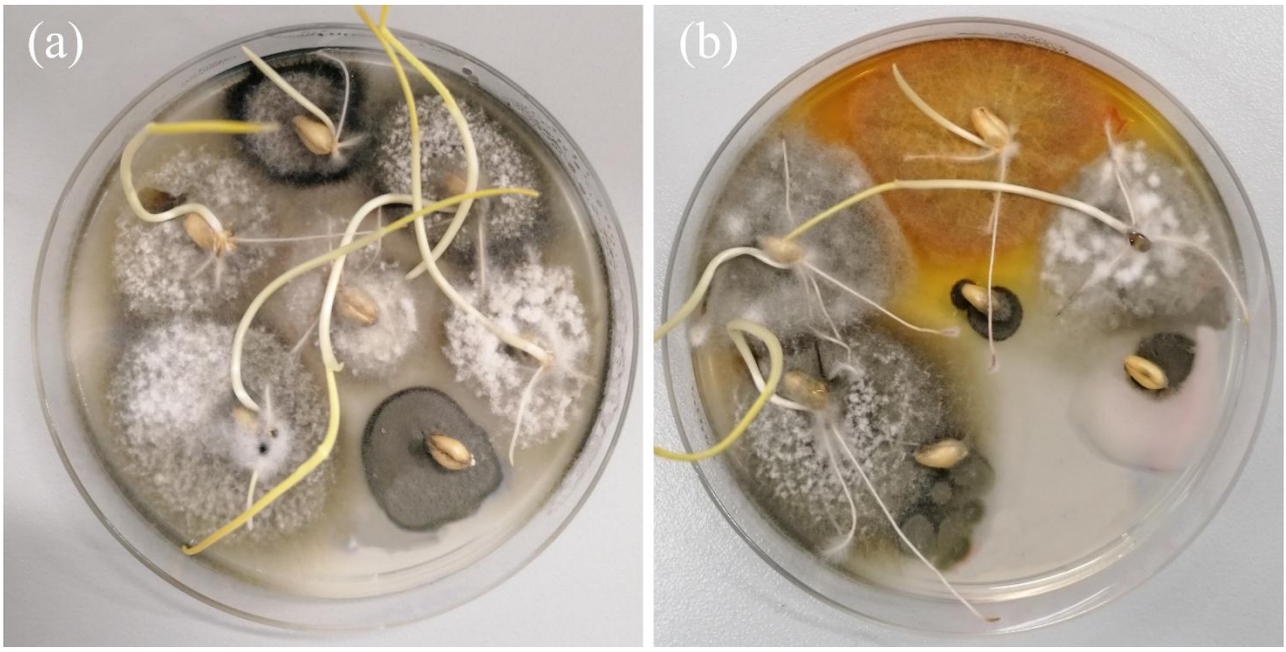


Figure S2. Variability of endogenous fungal contaminations of wheat kernels used during greenhouse experiment (a,b).

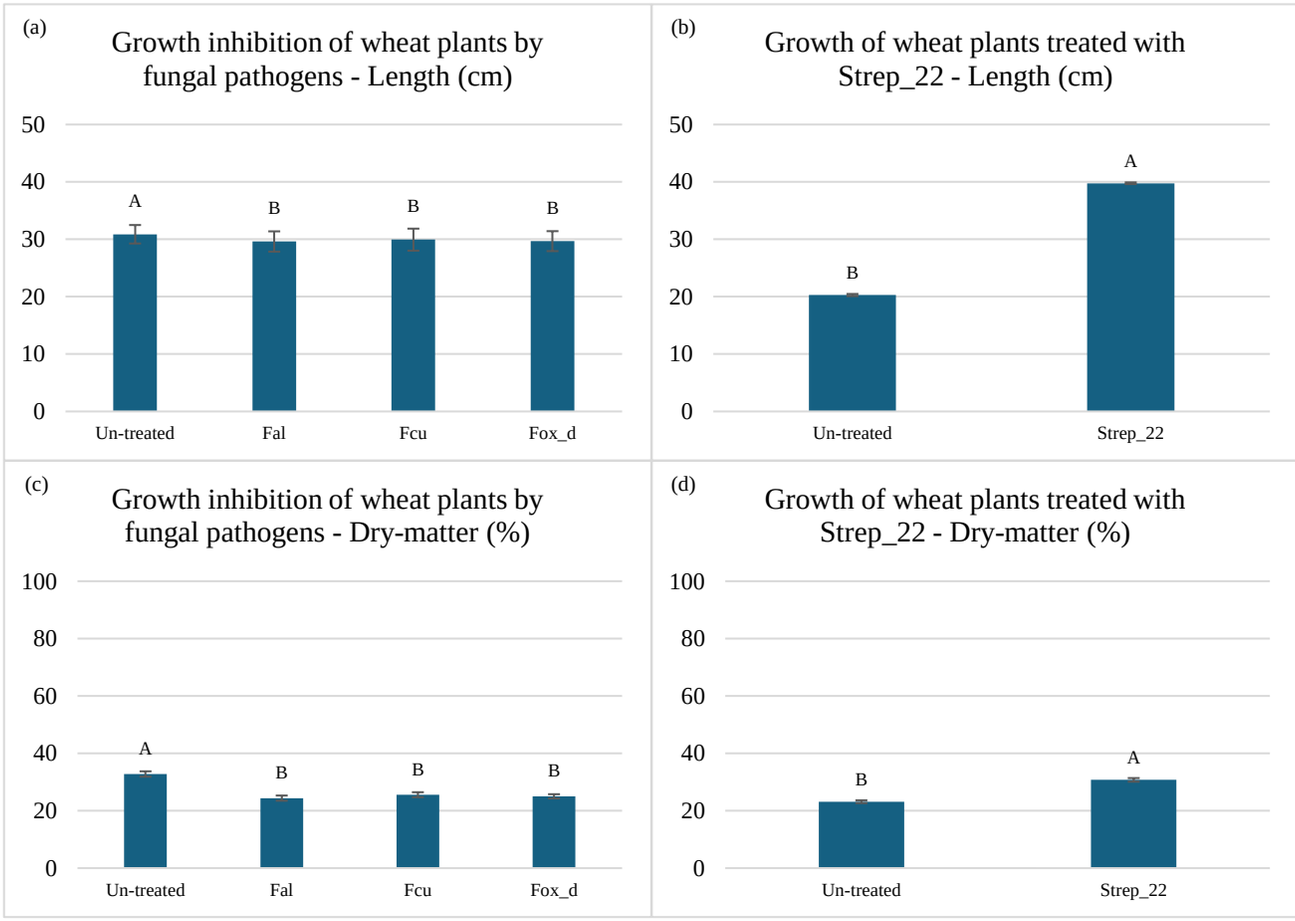


Figure S3. Effect of different treatments on the growth parameters of wheat plants: (a) reduction in final plant length caused by fungal pathogens; (b) promotion of shoot elongation by Strep_22; (c) reduction in biomass accumulation due to fungal pathogens, expressed as dry-matter percentage (%); (d) increase in dry-matter in plants treated with Strep_22. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Data were analysed using One-way ANOVA followed by Tukey's test ($p < 0.01$); means sharing the same capital letter are not significantly different.

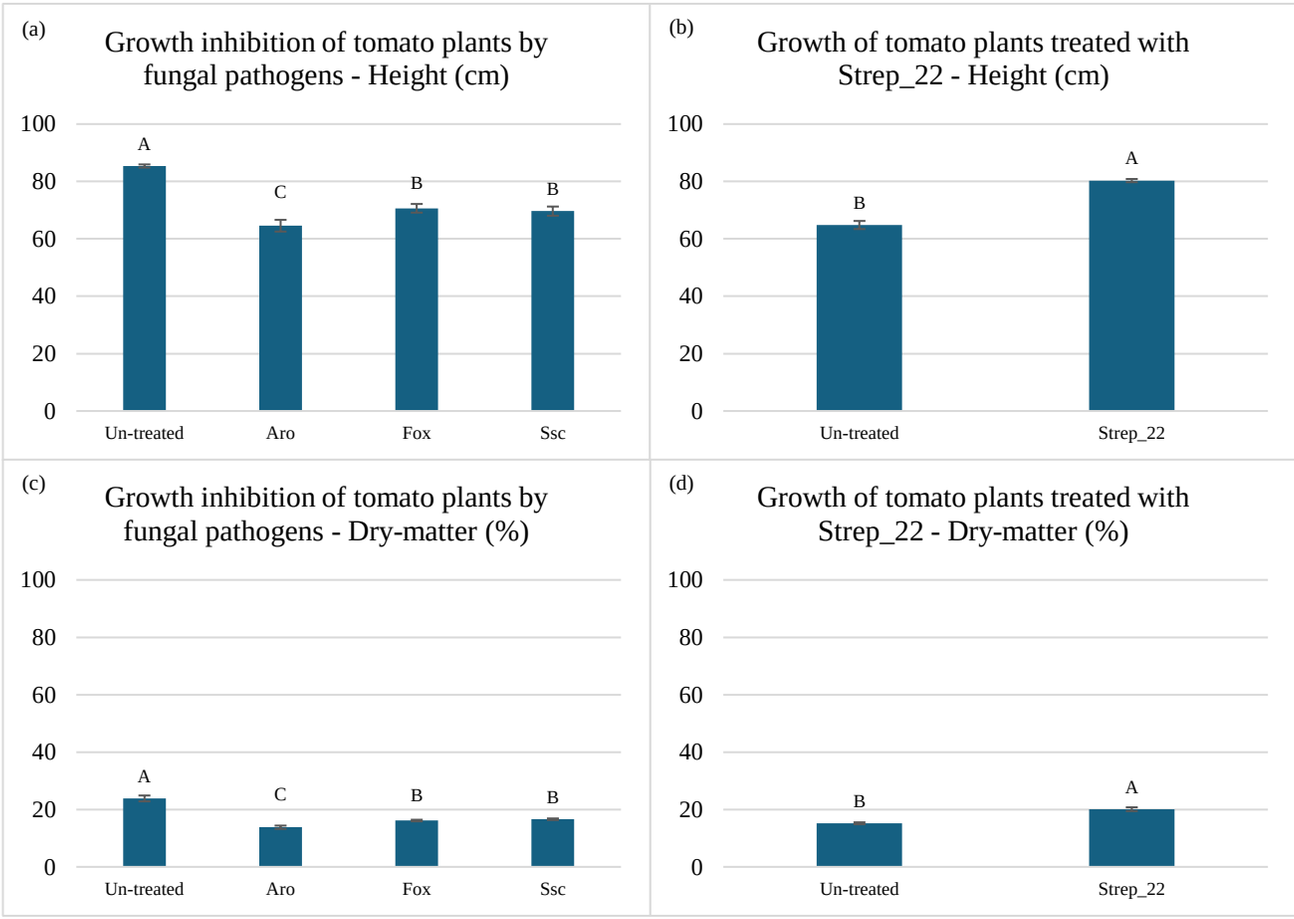


Figure S4. Effect of different treatments on the growth parameters of tomato plants: (a) reduction in final plant height caused by fungal pathogens; (b) promotion of shoot elongation by Strep_22; (c) reduction in biomass accumulation of tomato plants due to fungal pathogens, expressed as dry-matter percentage (%); (d) increase in dry-matter in plants treated with Strep_22. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. Data were analysed using One-way ANOVA followed by Tukey's test ($p< 0.01$); means sharing the same capital letter are not significantly different.

Table S1. Inhibiting activity of *Streptomyces* strains in dual culture in *in vitro* conditions.

Treatment	Inhibiting activity by S*treptomyces* strains (IA; %)	
	Mean±SE	**Min-Max**
Strep_22	91.52±2.03* A§	55.45-100.00
Strep_23	34.64±5.30 C	0.00-82.73
Strep_24	85.61±1.81 B	56.36-100.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova analysis, Tukey Test ($p< 0.01$).

Table S2. Sensitivity of the fungal species subjected to inhibiting activity of *Streptomyces* strains in dual culture in *in vitro* conditions.

Fungal species	Sensitivity of fungal species to *Streptomyces* strains (IA; %)	
	Mean±SE	Min-Max
Aro	61.21±13.20* F§	0.00-100.00
Fal	78.03±3.50 C	63.64-92.05
Fav	87.58±3.14 B	74.55-100.00
Fcu	59.55±12.70 G	0.00-90.91
Fox	78.94±4.91 C	56.36-94.55
Fox_d	68.79±5.30 D	56.36-93.64
Fso	68.18±11.88 D	12.73-100.00
Rso	47.57±10.87 H	0.00-87.27
Ssc	66.67±14.21 E	0.00-100.00
Vda	89.40±1.49 A	82.73-94.55

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fal: *Fusarium algeriense*; Fav: *F. avenaceum*; Fcu: *F. culmorum*; Fox: *F. oxysporum*; Fox_d: *F. oxysporum* f.sp. *dianthi*; Fso: *F. solani*; Rso; *Rhizoctonia solani*; Ssc: *Sclerotinia sclerotiorum*; Vda: *Verticillium dahliae*. One-way Anova analysis, Tukey Test ($p< 0.01$).

Table S3. Effect of interaction between inoculated fungal species and *Streptomyces* strains by inhibition of fungal growth under *in vitro* conditions.

Fungal species	Interaction between fungi and *Streptomyces* strain by inhibiting activity (IA; %)					
	Strep_22		Strep_23		Strep_24	
	Mean±SE	Min-Max	Mean±SE	Min-Max	Min-Max	Min-Max
Aro	100.00±0.00* A§	100.00-100.00	0.00±0.00 O	0.00-0.00	83.64±0.00 I	83.64-83.64
Fal	92.05±0.00 CD	92.05-92.05	63.64±0.00 L	63.64-63.64	78.41±0.66 J	77.27-79.55
Fav	100.00±0.00 A	100.00-100.00	74.55±0.00 K	74.55-74.55	88.18±0.52 FGH	87.27-89.09
Fcu	89.09±0.00 EFG	89.09-89.09	0.00±0.00 O	0.00-0.00	89.55±0.79 EF	88.18-90.91
Fox	94.10±0.26 B	93.64-94.55	56.36±0.00 M	56.36-56.36	86.36±0.52 H	85.45-87.27
Fox_d	93.64±0.00 BC	93.64-93.64	56.36±0.00 M	56.36-56.36	56.36±0.00 M	56.36-56.36
Fso	100.00±0.00 A	100.00-100.00	12.73±0.00 N	12.73-12.73	91.82±1.57 CD	89.09-94.55
Rso	55.45±0.00 M	55.45-55.45	0.00±0.00 O	0.00-0.00	87.27±0.00 GH	87.27-87.27
Ssc	100.00±0.00 A	100.00-100.00	0.00±0.00 O	0.00-0.00	100.00±0.00 A	100.00-100.00
Vda	90.91±0.00 DE	90.91-90.91	82.73±0.00 I	82.73-82.73	94.55±0.00 B	94.55-94.55

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fal: *Fusarium algeriense*; Fav: *F. avenaceum*; Fcu: *F. culmorum*; Fox: *F. oxysporum*; Fox_d: *F. oxysporum* f.sp. *dianthi*; Fso: *F. solani*; Rso; *Rhizoctonia solani*; Ssc: *Sclerotinia sclerotiorum*; Vda: *Verticillium dahliae*. Factorial Anova, Tukey Test ($p< 0.01$).

Table S4. Fungal growth kinetics based on parameter Δτ (prolongation of τ referred to PDA) (hours) by *Streptomyces* strains.

Treatment	Inhibition performed by *Streptomyces* strains of the pathogen growth expressed as Δτ	
	Mean±SE	Min-Max
Strep_22	101.84±41.85* A§	-143.31-427.31
Strep_23	-5.21±11.18 B	-123.82-139.72
Strep_24	-5.32±28.70 B	-175.17-425.60
Control	0.00±1.48 B	-18.10-0.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S5. Reduction of fungal growth kinetics based on parameter Δτ (prolongation of τ referred to PDA) by the Streptomyces strains.

Fungal species	Slowing of fungal growth by *Streptomyces* strains expressed as Δτ	
	Mean±SE	**Min-Max**
Aro	105.46±56.03* F§	-5.46-427.31
Fal	-38.02±13.89 C	-104.30-48.28
Fav	-9.28±47.87 B	-175.17-244.69
Fcu	7.44±13.59 G	-41.74-90.12
Fox	-30.86±13.68 C	-118.05-33.42
Fox_d	-34.48±10.06 D	-89.99-2.56
Fso	108.74±48.39 D	-34.24-376.11
Rso	-3.18±2.25 H	-18.69-4.01
Ssc	212.80±64.16 E	0.00-425.60
Vda	-90.35±18.40 A	-147.34-18.10

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fal: *Fusarium algeriense*; Fav: *F. avenaceum*; Fcu: *F. culmorum*; Fox: *F. oxysporum*; Fox_d: *F. oxysporum* f.sp. *dianthi*; Fso: *F. solani*; Rso; *Rhizoctonia solani*; Ssc: *Sclerotinia sclerotiorum*; Vda: *Verticillium dahliae*. One-way Anova, Tukey Test ($p< 0.01$).

Table S6. Wheat root rot caused by inoculated fungal pathogens during greenhouse experiment.

Fungal species	Disease incidence by inoculated fungi on wheat root (MkI; %)	
	Mean±SE	Min-Max
Un-treated	29.33±4.82* C§	0.00-100.00
Fal	57.43±5.55 A	1.79-90.91
Fcu	42.27±5.43 B	5.75-96.91
Fox_d	47.86±6.35 AB	0.00-100.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. One-way Anova, Tukey Test ($p< 0.01$).

Table S7. Phytosanitary status of wheat roots treated with Strep_22 during greenhouse experiment**.**

Treatment	Wheat root protection performed by Strep_22 (MkI; %)	
	Mean±SE	Min-Max
Un-treated	68.80±2.53* A§	20.56-100.00
Strep_22	19.64±2.86 B	0.00-100.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S8. Interaction between inoculated fungal pathogens and Strep_22 expressed as disease incidence on wheat root during greenhouse experiment.

Treatment	Effect of interaction Strep_22 *vs* fungus on disease incidence on root (MkI; %)	
	Mean±SE	**Min-Max**
Un-treated	47.79±5.85[ϕ] B[§]	20.56-100.00
Strep_22	10.87±4.06 D	0.00-57.58
Fal	81.76±1.17 A	69.64-90.91
Fal*Strep_22	33.09±6.85 BC	1.79-75.00
Fcu	69.60±4.12 A	38.33-96.91
Fcu*Strep_22	14.94±2.27 CD	5.57-35.90
Fox_d	79.06±3.26 A	42.59-96.79
Fox_d*Strep_22	19.65±7.06 CD	0.00-100.00

[ϕ]Mean of minimal squares, standard error; [§]Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Factorial Anova, Tukey Test ($p < 0.01$).

Table S9. Re-isolation rates of inoculated fungal pathogens from treated and un-treated wheat roots. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*.

Fungal species	**Re-Isolation frequency – R-IF (%)**							
	Un-treated	**Strep_22**	**Fal**	**Fal*Strep_22**	**Fcu**	**Fcu*Strep_22**	**Fox_d**	**Fox_d*Strep_22**
Fusarium algeriense	0 (0.00%)	0 (0.00%)	13 (27.08%)	3 (6.25%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
F. culmorum	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	12 (25.00%)	2 (4.17%)	0 (0.00%)	0 (0.00%)
F. oxysporum f.sp. *dianthi*	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	11 (22.92%)	1 (2.08%)
Alternaria spp.	11 (22.92%)	2 (4.17%)	13 (27.08%)	3 (6.25%)	14 (29.17%)	3 (6.25%)	15 (31.25%)	4 (8.33%)
Apergillus spp.	6 (12.50%)	0 (0.00%)	8 (16.67%)	0 (0.00%)	3 (6.25%)	0 (0.00%)	9 (18.75%)	0 (0.00%)
Cladosporium spp.	1 (2.08%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (2.08%)	0 (0.00%)
Epicoccum spp.	3 (6.25%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (2.08%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Fusarium spp.	11 (22.92%)	3 (6.25%)	1 (2.08%)	0 (0.00%)	3 (6.25%)	3 (6.25%)	5 (10.42%)	2 (4.17%)
Penicillium spp.	3 (6.25%)	0 (0.00%)	1 (2.08%)	0 (0.00%)	1 (2.08%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Phoma spp.	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	3 (6.25%)	0 (0.00%)
Stemphylium spp.	4 (8.33%)	0 (0.00%)	2 (4.17%)	0 (0.00%)	3 (6.25%)	0 (0.00%)	3 (6.25%)	0 (0.00%)
Trichothecium spp.	1 (2.08%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	4 (8.33%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Sterile mycelium	8 (16.67%)	0 (0.00%)	7 (14.58%)	0 (0.00%)	6 (12.5%)	0 (0.00%)	1 (2.08%)	0 (0.00%)
Subtotal only fungi	***48 (100.00%)***	***5 (10.42%)***	***45 (93.75%)***	***6 (12.5%)***	***47 (97.92%)***	***8 (16.67%)***	***48 (100.00%)***	***7 (14.58%)***
Bacteria	0 (0.00%)	0 (0.00%)	1 (2.08%)	3 (6.25%)	1 (2.08%)	2 (4.17%)	0 (0.00%)	1 (2.08%)
No gwowth	0 (0.00%)	43 (89.58%)	2 (4.17%)	39 (81.25%)	0 (0.00%)	38 (79.17%)	0 (0.00%)	40 (83.33%)
Total	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***

Table S10. Foliar health after fungal artificial inoculations at soil hosting wheat plants during greenhouse experiment.

Fungal species	Effect of the root infection by fungi on leaves (MkI; %)			
	First leaf		Second leaf	
	Mean±SE	Min-Max	Mean±SE	Min-Max
Un-treated	24.20±3.60* A§	0.00-51.23	5.53±1.21 B	0.00-19.00
Fal	28.78±2.96 A	1.67-52.98	5.67±1.09 B	0.00-19.23
Fcu	23.99±3.41 A	0.56-55.13	5.22±1.32 B	0.00-21.60
Fox_d	27.62±2.47 A	1.72-44.87	5.46±1.09 B	0.00-17.31

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. One-way Anova, Tukey Test ($p< 0.01$).

Table S11. Foliar phytosanitary status after Strep_22 treatments on wheat roots artificially inoculated with fungal species during greenhouse experiment.

Treatment	Effect of wheat root protection by Strep_22 on leaves (MkI; %)			
	First leaf		Second leaf	
	Mean±SE	Min-Max	Mean±SE	Min-Max
Un-treated	41.50±1.08* A§	11.67-55.13	10.47±0.76 B	0.56-21.60
Strep_22	10.80±1.09 B	0.00-28.89	0.47±0.13 C	0.00-7.78

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S12. Effect on wheat leaves of interaction between Strep_22 and inoculated fungal pathogens during greenhouse experiment.

	Effect of interaction of Strep_22 *vs* fungus on leaves disease incidence (MkI; %)			
	First leaf		**Second leaf**	
Treatment	**Mean±SE**	**Min-Max**	**Mean±SE**	**Min-Max**
Un-treated	4.78±1.33[ϕ] C[§]	0.00-15.00	10.72±1.58 A	0.56-18.00
Strep_22	38.92±3.81 A	11.67-55.13	0.34±0.09 B	0.00-1.11
Fal	9.06±1.93 BC	0.56-20.83	10.47±1.28 A	4.17-19.23
Fal*Strep_22	39.60±0.87 A	32.69-44.87	0.86±0.47 B	0.00-7.78
Fcu	13.72±2.13 B	1.67-27.78	10.07±2.03 A	0.56-21.60
Fcu*Strep_22	43.63±1.24 A	36.11-51.23	0.37±0.11 B	0.00-1.28
Fox_d	15.63±2.30 B	1.72-28.89	10.60±1.18 A	3.09-17.31
Fox_d*Strep_22	43.85±1.15 A	36.31-52.98	0.32±0.10 B	0.00-1.15

[ϕ]Mean of minimal squares, standard error; [§]Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Factorial Anova, Tukey Test ($p< 0.01$).

Table S13. Effect of fungal infection on the final length of wheat plants during greenhouse experiment.

Fungal species	Influence of fungal pathogens on wheat growth – Length (cm)	
	Mean±SE	Min-Max
Un-treated	30.88±1.61* A§	20.50-40.79
Fal	29.62±1.77 B	18.60-40.84
Fcu	29.94±1.92 B	17.80-42.19
Fox_d	29.68±1.74 B	18.60-42.13

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. One-way Anova, Tukey Test ($p< 0.01$).

Table S14. Plant growth promotion by Strep_22 treatments on wheat during greenhouse experiment.

Treatment	Promotion by Strep_22 on wheat length (cm)	
	Mean±SE	**Min-Max**
Un-treated	20.29±0.19* B§	17.80-25.20
Strep_22	39.77±0.15 A	37.06-42.19

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S15. Effect of fungal infection on bioaccumulation of wheat plants during greenhouse experiment.

Fungal species	Influence of fungal pathogens on wheat growth – Dry-matter (%)	
	Mean±SE	Min-Max
Un-treated	32.81±0.94* A§	26.11-43.05
Fal	24.40±0.90 B	16.47-32.88
Fcu	25.59±0.87 B	18.00-32.91
Fox_d	25.03±0.74 B	20.10-32.34

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. One-way Anova, Tukey Test ($p< 0.01$).

Table S16. Bioaccumulation promotion by Strep_22 treatments on wheat plants during greenhouse experiment.

Treatment	Promotion of wheat growth by Strep_22 – Dry-matter (%)	
	Mean±SE	**Min-Max**
Un-treated	23.16±0.49* B§	16.47-31.99
Strep_22	30.76±0.63 A	22.21-43.04

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S17. Wheat plant protection expressed as growth promotion by Strep_22 against artificial fungal infections under greenhouse experiment.

Treatment	Effect of interaction between Strep_22 and fungus on growth of wheat plant – Length (cm)	
	Mean±SE	Min-Max
Un-treated	22.02±0.41[ϕ] B[§]	20.50-25.20
Strep_22	39.74±0.21 A	37.97-40.79
Fal	19.80±0.23 C	18.60-21.50
Fal*Strep_22	39.43±0.24 A	37.85-40.84
Fcu	19.32±0.21 C	17.80-20.50
Fcu*Strep_22	40.56±0.36 A	37.06-42.19
Fox_d	20.03±0.27 C	18.60-22.00
Fox_d*Strep_22	39.43±0.28 A	37.66-42.13

[ϕ]Mean of minimal squares, standard error; [§]Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Factorial Anova, Tukey Test ($p < 0.01$).

Table S18. Bioaccumulation of wheat plants as dry-matter by interaction between Strep_22 and fungi during greenhouse experiment.

Treatment	Effect of interaction between Strep_22 and fungus on growth of wheat plant - Dry-matter (%)	
	Mean±SE	Min-Max
Un-treated	28.27±0.52[ϕ] B[§]	26.11-31.99
Strep_22	37.36±0.78 A	32.67-43.05
Fal	20.10±0.47 C	16.47-22.51
Fal*Strep_22	28.71±0.82 B	22.52-32.88
Fcu	22.55±0.96 C	18.00-29.35
Fcu*Strep_22	28.62±0.97 B	22.74-32.91
Fox_d	21.72±0.40 C	20.10-25.27
Fox_d*Strep_22	28.34±0.80 B	22.21-32.34

[ϕ]Mean of minimal squares, standard error; [§]Different capital letters indicate the significance among means. Fal: *Fusarium algeriense*; Fcu: *F. culmorum*; Fox_d: *F. oxysporum* f.sp. *dianthi*. Factorial Anova, Tukey Test ($p< 0.01$).

Table S19. Tomato root rot caused by inoculated fungal pathogens during greenhouse experiment.

Fungal species	Disease incidence caused by inoculated fungi on tomato root (MkI; %)	
	Mean±SE	Min-Max
Un-treated	1.04±0.59* C§	0.00-16.67
Aro	29.17±5.17 A	0.00-100.00
Fox	13.89±2.95 B	0.00-66.67
Ssc	14.93±2.18 B	0.00-50.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. One-way Anova, Tukey Test ($p< 0.01$)

Table S20. Phytosanitary status of tomato roots treated with Strep_22 during greenhouse experiment**.**

Treatment	Tomato root protection performed by Strep_22 (MkI; %)	
	Mean±SE	Min-Max
Un-treated	23.44±2.71* A§	0.00-100.00
Strep_22	6.08±1.78 B	0.00-100.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S21. Interaction between inoculated fungal pathogens and Strep_22 expressed as disease incidence on tomato root during greenhouse experiment.

Treatment	Effect of interaction Strep_22 *vs* fungus on disease incidence on tomato root (MkI; %)	
	Mean±SE	Min-Max
Un-treated	0.69±0.15[ϕ] D[§]	0.00-16.67
Strep_22	1.39±0.26 D	0.00-16.67
Aro	43.06±7.37 A	0.00-100.00
Aro*Strep_22	15.29±6.18 BCD	0.00-100.00
Fox	27.08±4.47 AB	0.00-66.67
Fox*Strep_22	0.69±0.15 D	0.00-16.67
Ssc	22.92±2.62 BC	0.00-50.00
Ssc*Strep_22	6.94±2.64 CD	0.00-50.00

[ϕ]Mean of minimal squares, standard error; [§]Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. Factorial Anova, Tukey Test ($p< 0.01$).

Tabe S22. Re-isolation rates of inoculated fungal pathogens from treated and un-treated tomato root. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*.

Funga species	Re-Isolation frequency – R-IF (%)							
	Un-treated	**Strep_22**	**Aro**	**Aro*Strep_22**	**Fox**	**Fox*Strep_22**	**Ssc**	**Ssc*Strep_22**
Agroathelia rolfsii	0 (0.00%)	0 (0.00%)	5 (10.42%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Fusarium oxysporum	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	4 (8.33%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Sclerotinia sclerotiorum	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	3 (6.25%)	0 (0.00%)
Acremonium spp.	2 (4.17%)	1 (2.08%)	1 (2.08%)	0 (0.00%)	1 (2.08%)	0 (0.00%)	2 (4.17%)	1 (2.08%)
Alternaria spp.	2 (4.17%)	0 (0.00%)	2 (4.17%)	1 (2.08%)	1 (2.08%)	1 (2.08%)	1 (2.08%)	1 (2.08%)
Apergillus spp.	1 (2.08%)	1 (2.08%)	2 (4.17%)	0 (0.00%)	1 (2.08%)	1 (2.08%)	1 (2.08%)	1 (2.08%)
Fusarium spp.	1 (2.08%)	0 (0.00%)	0 (0.00%)	1 (2.08%)	0 (0.00%)	2 (4.17%)	0 (0.00%)	0 (0.00%)
Penicillium spp.	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Phoma spp.	13 (27.08%)	1 (2.08%)	12 (25.00%)	1 (2.08%)	20 (41.67%)	1 (2.08%)	10 (20.83%)	1 (2.08%)
Rhizopus spp.	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Stemphylim spp.	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (2.08%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Thricothecium spp.	2 (4.17%)	0 (0.00%)	3 (6.25%)	1 (2.08%)	0 (0.00%)	1 (2.08%)	1 (2.08%)	1 (2.08%)
Sterile mycelium	3 (6.25%)	1 (2.08%)	7 (14.58%)	1 (2.08%)	8 (16.67%)	0 (0.00%)	1 (2.08%)	1 (2.08%)
Subtotal only fungi	***24 (50.00%)***	***4 (8.33%)***	***32 (66.67%)***	***6 (12.50%)***	***35 (72.92%)***	***6 (12.50%)***	***19 (39.58%)***	***6 (12.50%)***
Bacteria	5 (10.42%)	0 (0.00%)	2 (4.17%)	0 (0.00%)	2 (4.17%)	0 (0.00%)	5 (10.42%)	0 (0.00%)
No growth	19 (39.58%)	44 (91.67%)	14 (29.17%)	42 (87.50%)	11 (22.92%)	42 (87.50%)	24 (50.00%)	42 (87.50%)
Total	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***	***48 (100.00%)***

Table S23. Collar and aerial parts health after fungal artificial inoculations at soil hosting tomato plants during greenhouse experiment.

Fungal species	Effect of the root infection by fungi on collar and aerial parts (MkI; %)			
	Collar		Aerial parts	
	Mean±SE	Min-Max	Mean±SE	Min-Max
Un-treated	0.00±0.00* C§	0.00-0.00	16.67±2.85 B	0.00-66.67
Aro	20.83±4.34 B	0.00-83.33	36.81±3.78 A	0.00-83.33
Fox	4.51±1.77 C	0.00-50.00	26.74±3.17 AB	0.00-66.67
Ssc	2.08±0.94 C	0.00-33.33	23.96±2.89 B	0.00-66.67

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. One-way Anova, Tukey Test ($p< 0.01$).

Table S24. Collar and aerial parts phytosanitary status after Strep_22 treatments on tomato roots artificially inoculated with fungal species during greenhouse experiment.

Treatment	Effect of tomato root protection by Strep_22 on collar and aerial parts (MkI; %)			
	Collar		Aerial parts	
	Mean±SE	Min-Max	Mean±SE	Min-Max
Un-treated	10.94±2.26* B§	11.67-55.13	36.11±2.72 A	0.00-83.33
Strep_22	2.78±1.27 C	0.00-28.89	15.97±1.28 B	0.00-83.33

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S25. Effect on tomato collar and aerial parts of interaction between Strep_22 and inoculated fungal pathogens during greenhouse experiment.

	Effect of interaction of Strep_22 *vs* fungus on collar and aerial parts disease incidence (MkI; %)			
	Collar		**Aereial parts**	
Treatment	**Mean±SE**	**Min-Max**	**Mean±SE**	**Min-Max**
Un-treated	0.00±0.00$^{\phi}$ F§	0.00-0.00	18.06±5.39 CDE	0.00-66.67
Strep_22	0.00±0.00 F	0.00-0.00	15.28±1.99 DEF	0.00-50.00
Aro	33.33±6.34 BC	0.00-83.33	54.86±3.95 A	16.67-83.33
Aro*Strep_22	8.33±4.81 DEF	0.00-83.33	18.75±3.80 CD	0.00-83.33
Fox	9.03±3.32 DEF	0.00-50.00	38.19±4.76 AB	0.00-66.67
Fox*Strep_22	0.00±0.00 F	0.00-0.00	15.28±2.64 DEF	0.00-50.00
Ssc	1.39±1.39 EF	0.00-33.33	33.33±5.02 BC	0.00-66.67
Ssc*Strep_22	2.78±1.3 DEF	0.00-16.67	14.58±1.15 DEF	0.00-16.67

$^{\phi}$Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. Factorial Anova, Tukey Test ($p< 0.01$).

Table S26. Effect of fungal infection on the final height of tomato plants during greenhouse experiment.

Fungal species	Influence of fungal pathogens on tomato growth – Height (cm)	
	Mean±SE	Min-Max
Un-treated	85.39±0.55* A§	79.50-91.00
Aro	64.58±2.04 C	0.00-82.00
Fox	70.65±1.51 B	57.00-87.50
Ssc	69.66±1.60 B	52.50-85.50

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. One-way Anova, Tukey Test ($p< 0.01$).

Table S27. Plant growth promotion by Strep_22 treatments on tomato during greenhouse experiment.

Treatment	Promotion by Strep_22 on tomato height (cm)	
	Mean±SE	Min-Max
Un-treated	64.83±1.41 B	0.00-91.00
Strep_22	80.31±0.56 A	70.00-91.00

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S28. Effect of fungal infection on bioaccumulation of tomato plants during greenhouse experiment.

Fungal species	Influence of fungal pathogens on tomato growth – Dry-matter (%)	
	Mean±SE	Min-Max
Un-treated	23.85±1.03* A§	13.84-35.34
Aro	13.81±0.58 C	6.84-20.52
Fox	16.15±0.28 B	10.64-19.89
Ssc	16.60±0.28 B	12.08-21.91

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. One-way Anova, Tukey Test ($p < 0.01$).

Table S29. Bioaccumulation promotion by Strep_22 treatments on tomato plants during greenhouse experiment.

Treatment	Promotion of tomato growth by Strep_22 – Dry-matter (%)	
	Mean±SE	**Min-Max**
Un-treated	15.18±0.35* B§	6.84-21.23
Strep_22	20.10±0.66 A	10.67-35.34

*Mean of minimal squares, standard error; §Different capital letters indicate the significance among means. One-way Anova, Tukey Test ($p< 0.01$).

Table S30. Tomato plant protection expressed as growth promotion by Strep_22 against artificial fungal infections under greenhouse experiment.

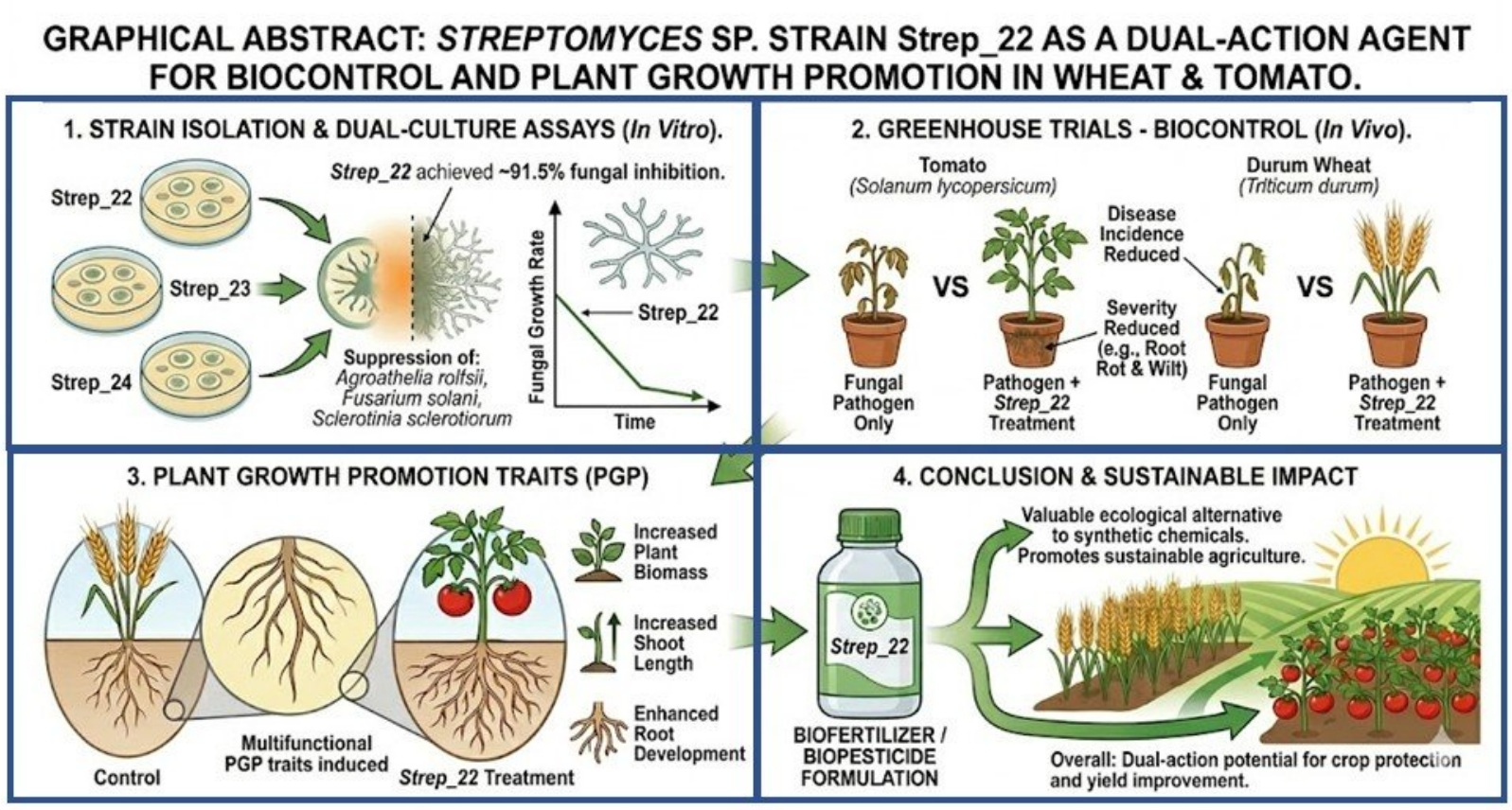
Treatment	Effect of interaction between Strep_22 and fungus on growth of tomato plant – Height (cm)	
	Mean±SE	Min-Max
Un-treated	84.94±0.87[ϕ] AB[§]	79.50-91.00
Strep_22	85.83±0.69 A	79.50-91.00
Aro	54.13±2.59 E	0.00-61.50
Aro*Strep_22	75.04±0.89 C	70.00-82.00
Fox	60.94±0.64 D	57.00-67.50
Fox*Strep_22	80.35±0.87 B	75.00-87.50
Ssc	59.31±0.78 D	52.50-65.00
Ssc*Strep_22	80.00±0.76 BC	74.50-85.50

[ϕ]Mean of minimal squares, standard error; [§]Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. Factorial Anova, Tukey Test ($p< 0.01$).

Table S31. Bioaccumulation of tomato plants as dry-matter by interaction between Strep_22 and fungi during greenhouse experiment.

Treatment	Effect of the interaction between Strep_22 and fungus on growth of tomato plant - Dry-matter (%)	
	Mean±SE	Min-Max
Un-treated	17.11±0.46 ϕ B§	13.84-21.23
Strep_22	30.58±0.45 A	26.39-35.34
Aro	10.67±0.52 C	6.84-16.75
Aro*Strep_22	16.95±0.46 B	10.67-20.52
Fox	16.56±0.41 B	10.64-19.89
Fox*Strep_22	15.73±0.37 B	11.76-19.27
Ssc	16.19±0.42 B	12.08-19.52
Ssc*Strep_22	17.02±0.37 B	12.61-21.91

ϕMean of minimal squares, standard error; §Different capital letters indicate the significance among means. Aro: *Agroathelia rolfsii*; Fox: *Fusarium oxysporum*; Ssc: *Sclerotinia sclerotiorum*. Factorial Anova, Tukey Test ($p< 0.01$).


GRAPHICAL ABSTRACT: STREPTOMYCES SP. STRAIN Strep_22 AS A DUAL-ACTION AGENT FOR BIOCONTROL AND PLANT GROWTH PROMOTION IN WHEAT & TOMATO.
1. STRAIN ISOLATION & DUAL-CULTURE ASSAYS (In Vitro).
Strep_22
Strep_23
Strep_24
Strep_22 achieved ~91.5% fungal inhibition.
Suppression of:
Agroathelia rolfsii,
Fusarium solani,
Sclerotinia sclerotiorum
Fungal Growth Rate
Time
Strep_22
2. GREENHOUSE TRIALS - BIOCONTROL (In Vivo).
Tomato
(Solanum lycopersicum)
Durum Wheat
(Triticum durum)
VS
VS
Disease Incidence Reduced
Severity Reduced (e.g., Root Rot & Wilt)
Fungal Pathogen Only
Pathogen + Strep_22 Treatment
Fungal Pathogen Only
Pathogen + Strep_22 Treatment
3. PLANT GROWTH PROMOTION TRAITS (PGP)
Increased Plant Biomass
Increased Shoot Length
Enhanced Root Development
Control
Multifunctional PGP traits induced
Strep_22 Treatment
4. CONCLUSION & SUSTAINABLE IMPACT
Strep_22
BIOFERTILIZER / BIOPESTICIDE FORMULATION
Valuable ecological alternative to synthetic chemicals.
Promotes sustainable agriculture.
Overall: Dual-action potential for crop protection and yield improvement.

Objective 7. Genomic and metabolomic characterization of the most promising *Streptomyces* strain (Strep_22).

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Genomic and matabolomic characterization of *Streptomyces syringium* with biofungicide potential

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Abstract

Fungal pathogens, weeds, and insects represent a serious threat to global agriculture. To prevent food losses, modern agriculture is increasingly projected to use novel, sustainable tools, such as plant-derived products and microorganisms. Among the latter, microorganisms with evaluable antagonistic activity, such as *Streptomyces* spp., offer a potential solution for pest management. This genus belongs to the phylum *Actinomycetota* (formerly *Actinobacteria*), which represents one of the largest, oldest, and most diverse phyla within the domain Bacteria. Phylogenetically, they constitute an ancient, deeply branching lineage, while the diversity of their genome makes molecular identification complex, it also reflects the wide range of secondary metabolites produced by this genus. In fact, the antifungal, herbicide, and insecticide activities of *Streptomyces* spp. are mainly related to these secondary metabolites. Thus, a genomic and metabolomic characterization of the *Streptomyces* sp. strain Strep_22 was performed, as this strain demonstrated strong antifungal and plant-growth-promoting activity under both *in vitro* and *in vivo* conditions. The genomic characterization revealed that the strain belongs to *Streptomyces syringium*, which is closely related to *Streptomyces netropsis*. The genome of this species is rich in secondary metabolite biosynthetic gene clusters (smBGCs), which are clusters of genes involved in the production of secondary metabolites. Metabolite isolation and characterization confirmed the high potential of the strain. Among the secondary metabolites produced, the main compounds were identified as indole alkaloids (pimprinine, pimprinethine, and WS-30581 A), diketopiperazines (cyclo(Phe-Pro) and cyclo(Pro-Val)), and phthalates (diethyl phthalate). These compounds are reported to be effective against pests, confirming that *S. syringium*

Strep_22 is a valid candidate for the sustainable management of fungal pathogens. The bio-essays performed to assess antifungal potential from two total metabolomics extracts and two metabolites did not yield reliable results due to the small sample sizes available.

Keywords: *Streptomyces*, genomic identification, metabolomics characterization

1. Introduction

One of the biggest challenges for modern agriculture is to guarantee high food production while reducing chemical inputs. For decades, traditional agriculture based the management of biotic adversities, such as fungal pathogens, weeds, and insects, on the application of chemical and synthetic pesticides (Ayilara et al., 2023). These biotic adversities can lead to significant yield losses both in the open field and post-harvest, representing a serious concern for the increasing global demand for food (Gerken and Morrison, 2022; Ristaino et al., 2021). Although chemicals prevented heavy yield losses, their application has resulted in negative impacts on human health, the environment, and biodiversity. Their overuse is associated with chronic diseases, such as Alzheimer's disease and cancer, as well as neuro-developmental disorders, such as ADHD and autism (Mostafalou and Abdollahi, 2013; Xu et al., 2023; Yan et al., 2016). Exposure can occur through inhalation, specifically for spray pesticides, skin contact, and direct or indirect ingestion (Shekhar et al., 2024). In fact, chemical residues can contaminate the surface and inner tissues of fruits and vegetables, and are not totally removed even after washing (Bajwa and Sandhu, 2014). Contamination can also occur in animal-derived food, either through the ingestion of contaminated drinking water or via biomagnification along the food chain (Abdulrahman et al., 2023). The bioaccumulation of chemical pesticides can also harm non-target organisms, such as beneficial soil microorganisms, parasitoids, or pollinators (Swaine et al., 2025; Theenoor et al., 2024). For instance, the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) global assessment on pollinators, pollination, and food production identified pesticides as one of the major drivers of pollinator decline (Basu et al., 2024). Most agrochemicals negatively affect soil microbial functions and biochemical processes. The resulting alteration in the diversity and composition of the beneficial microbial community can be un-favourable to plant growth and development, either by reducing nutrient availability or by increasing disease incidence (Meena et al., 2020). To preserve human health, the environment, and biodiversity from pesticides, the EU enforces strict rules, ranging from setting maximum residue levels (MRLs) to banning harmful substances under Regulation (EC) No. 1107/2009 (EFSA, 2026a; EFSA, 2026b). Regulation (EC) No. 1107/2009 is the main European Union law governing the authorization, commercialization, and use of plant protection products

(PPPs), serving as a direct policy response to recent concerns regarding the impact of chemicals on human health and the environment. PPPs can be removed from the market based on scientific studies that confirm their negative impacts. Once an active ingredient is approved by the EU, individual commercial products containing that ingredient must be authorized by the respective member countries (EU, 2009). The active enforcement of Article 4 (Approval criteria) and the hazard-based "cut-offs" mandated by Regulation (EC) No 1107/2009 have led to the ban of many pesticides, including Carbendazim (2014), Linuron (2017), Clothianidin (2018), Imidacloprid (2018), Thiamethoxam (2018), Diquat (2019), Chlorpyrifos (2020), Chlorpyrifos-methyl (2020), Epoxiconazole (2020), Mancozeb (2021), Phosmet (2022), Benthiavalicarb (2024), Clofentezine (2024), Dimethomorph (2024), Metiram (2024), S-Metolachlor (2024), and Triflusulfuron-methyl (2024). The dangers associated with the use of synthetic pesticides have necessitated the alternative use of natural means, which are cheaper, environmentally friendly, and sustainable (Ayilara et al., 2023). Plant disease management is a key factor in the sustainability of farming systems. The main aim of sustainable management is to keep pest damage below economic thresholds, thereby avoiding unacceptable costs, labour, and environmental impacts. Integrated Pest Management (IPM) and biological control aim to manage fungal pathogens, weeds, and insects by partially or completely reducing the application of chemical pesticides, respectively. IPM relies on assembling complementary approaches depending on the phytosanitary status of the plant, the geographical location, and the pedoclimatic conditions. Within this framework, chemical pesticides should be considered as a last resort when agronomic, physical, and sustainable tools fail to achieve the required pest control. In contrast, biological control completely excludes the application of chemical pesticides, relying solely on biocontrol agents (BCAs) and natural substances (Spadaro and Gullino, 2019). Among fungal pathogens, diseases caused by soilborne pathogens cause heavy losses to many crops, resulting in seedling, vascular, and root rot diseases. Although soilborne pathogens include a highly diverse group of organisms, such as fungi, oomycetes, nematodes, viruses, and parasitic plants, they share key biological features. Specifically, they survive and operate within the soil matrix during all or at least a part of their life cycle. Consequently, they are heavily influenced by both the biotic and abiotic components of the soil, as well as by agronomic practices such as irrigation, tillage, manure application, and fertilization. They typically invade plants through belowground organs but can eventually reach the upper epigeal parts of the host (Katan, 2017). Healthy soils are fundamental to the sustainable management of soilborne fungal pathogens (Spadaro and Gullino, 2019). For instance, *Trichoderma virens* DM5 suppresses *Athelia rolfsii* (formerly *Agroathelia rolfsii*) via both mycoparasitism and antibiosis, producing antifungal compounds that inhibit pathogen growth (Thaiparambil et al., 2026). Furthermore, *Pseudomonas* spp. and non-pathogenic *Fusarium* spp. play

a key role in naturally occurring soils that are suppressive to Fusarium wilt. Specifically, the suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas* spp. has been attributed to the production of 2,4-diacetylphloroglucinol (Mazzola, 2002). Plant resilience against soilborne fungal pathogens can also be increased by enhancing host defence mechanisms, such as induced systemic resistance (ISR), or by promoting plant growth. *Trichoderma* spp. can elicit ISR in plants, triggering defence responses associated with processes such as strengthening the plant cell wall, regulating secondary metabolic pathways, and recognizing pathogen-associated molecular patterns (PAMPs). For example, *Trichoderma virens* DM5 enhances host vigour by increasing seed germination by 54.5% and plant survival rates by 70% under pathogen pressure through the activation of defence-associated pathways. Beyond fungi, bacterial groups belonging to *Actinomycetota*, *Bacteroidota*, *Bacillota* (*Firmicutes*), and *Pseudomonadota* (*Proteobacteria*) contribute to pathogen suppression through key functions, including nutrient cycling, siderophore production, and phytohormone modulation (Fierer, 2017; Thaiparambil et al., 2026). The application of bacteria belonging to the genus *Streptomyces* as biocontrol agents (BCAs) represents an alternative disease management strategy that reduces environmental impacts and is fully compatible with organic agriculture (LeBlanc, 2022). *Streptomyces* spp. are Gram-positive, spore-forming, filamentous bacteria belonging to the family *Streptomycetaceae*, classified under the phylum *Actinomycetota* (formerly *Actinobacteria*). These bacteria can be isolated from a broad range of habitats, including marine and terrestrial ecosystems, mangroves, and extreme environments. In agriculture, *Streptomyces* spp. serve as biocontrol agents against a wide range of plant pathogens, including both fungi and bacteria (Jahan et al., 2025). According to Carlucci et al. (2022), *Streptomyces albidoflavus* strain CARA17 is capable of controlling several soilborne fungal pathogens, such as *Athelia rolfsii, Fusarium oxysporum, Plectosphaerella ramiseptata, Sclerotinia sclerotiorum*, and *Verticillium dahliae* under *in vitro* conditions, as well as the same strain protects fennel plants from fungal infections under *in vivo* conditions. Various other *Streptomyces* spp. have been proven effective in controlling soilborne fungal pathogens; these include *S. aureofaciens, S. avermitilis, S. humidus, S. hygroscopicus, S. lividans, S. lydicus, S. olivaceoviridis, S. plicatus, S. roseoflavus,* and *S. violaceusniger* (Jahan et al., 2025). The genus *Streptomyces*, comprising over 900 identified species, is renowned for its remarkable metabolomic versatility and ecological adaptability. It produces a diverse range of bioactive natural compounds with substantial medical and agricultural importance. Their secondary metabolites, known for their broad-spectrum activity, are widely used in agriculture for the development of antibacterial, antifungal, and anthelmintic agents, playing a key role in biocontrol processes. In addition to secondary metabolites, *Streptomyces* spp. produce specialized antibiotics and chitinolytic enzymes, which make them uniquely effective biocontrol agents (Jahan et al., 2025).

Specific secondary metabolites, such as 32,33-didehydroroflamycoin, butyrolactol A, hygromycin A, nigericin, reveromycin E, and reveromycin E1 methyl ester, have been reported to be effective against a wide spectrum of fungal pathogens (Kawicha et al., 2024; Kim et al., 2019; Nguyen et al., 2021). Additionally, *Streptomyces* spp. synthesize various phytohormones, hydrolytic enzymes, and volatile organic compounds (VOCs) that contribute to ISR in plants. For instance, geosmin, the VOC responsible for the characteristic smell of wet soil (petrichor), is a bicyclic alcohol derivative of decalin produced by *Streptomyces* spp. This compound influences bacterial cooperation and competition, helping the bacteria adapt to diverse environments, including complex microbial communities and host environments (Jahan et al., 2025). Among other VOCs produced by *Streptomyces* spp., 2-ethyl-5-methylpyrazine, varepsilon-poly-L-lysine, and L-linalool are actively involved in the control of *Botrytis cinerea, Ceratocystis fimbriata, Colletotrichum gloeosporioides*, and *Penicillium expansum* (Boykova et al., 2023; Dow et al., 2023; Gong et al., 2022). These multifaceted mechanisms not only protect plants from phytopathogens but also actively promote plant growth. *Streptomyces* spp. have been shown to increase crop yield, enhance root nodulation, and produce indole-3-acetic acid (IAA). Furthermore, they can activate the jasmonic acid (JA) and salicylic acid (SA)-mediated signaling pathways, which ultimately trigger the development of ISR. Finally, hydrolytic enzymes such as cellulases, chitinases, and proteases produced by *Streptomyces* spp. play a dual role in both promoting plant growth and directly suppressing phytopathogens (Jahan et al., 2025). *Streptomyces* spp. genomes demonstrate high levels of internal homology, indicating that the genome of their last common ancestor was already complex. Importantly, each species is characterized by species-specific fingerprint proteins. Even among closely related species, there is high interspecies variability regarding chromosomal protein-coding genes, species-level core genes, accessory genes, and fingerprints. Notably, secondary metabolite biosynthetic gene clusters (smBGCs), carbohydrate-active enzymes (CAZymes), and protein-coding genes bearing the rare TTA codon demonstrate high intraspecies and interspecies variability, emphasizing the need for strain-specific genome mining (Nikolaidis et al., 2023). In this context, smBGCs are physically clustered groups of two or more genes in a genome that collectively encode the enzymes and pathways required to produce specific secondary metabolites (Abdelsalam et al., 2023). CAZymes comprise a diverse group of proteins that catalyze the assembly, modification, and breakdown of complex carbohydrates and glycoconjugates (Wardman et al., 2022). Lastly, TTA-codon-bearing refers to genes or mRNAs containing the UUA/TTA triplet; TTA is considered a rare codon in many organisms, especially in GC-rich bacteria such as *Streptomyces* spp. (Silov et al., 2021). Highly conserved genes, such as those specifying genus-level core proteins, tend to occur in the central region of the chromosome. Conversely, genes encoding proteins with evolutionarily volatile species-level fingerprints, smBGCs,

CAZymes, and TTA-codon-bearing genes are predominantly located toward the telomeric ends of the linear chromosome. Thus, the chromosomal arms emerge as the genomic regions primarily responsible for rapid adaptation at both the species and strain levels. Furthermore, a moderate but statistically significant correlation exists between the total number of CAZymes and three categories of smBGCs (siderophores, varepsilon-poly-L-lysine, and type III lanthipeptides) that are directly involved in bacterial competition (Nikolaidis et al., 2023).Investigation of the biosynthetic machinery in *Streptomyces* spp. provides critical insights into natural product biosynthesis and enables the application of combinatorial biosynthesis approaches to expand structural diversity and accelerate the discovery of novel bioactive compounds (Jahan et al., 2025). *Streptomyces* spp. produce small diffusible signaling molecules at nanomolar concentrations, which meticulously regulate the expression of these secondary metabolites and antibiotics. The signaling molecules discovered so far can be categorized into furans, gamma-butyrolactones, and gamma-butenolides (Arakawa, 2018). For instance, the genome of *Streptomyces rochei* S32, a strain reported to inhibit several fungal pathogens, consists of an 8,041,158-bp chromosome and two plasmids. A genome mining study revealed 2,205 genes involved in secondary metabolism, potentially leading to the synthesis of compounds including acivicin, corynebactin, phytosphingosine, indole-3-acetic acid (IAA), siderophores, and 29 phytohormone-related molecules (Wei et al., 2024). A single strain of *Streptomyces* isolated from disease-suppressive soil was found to contain up to 35 biosynthetic gene clusters (BGCs) encoding potential antimicrobial substances (Cha et al., 2016). Recently, three *Streptomyces* strains (Strep_22, Strep_23, and Strep_24) were tested against ten soilborne fungal pathogens under in vitro conditions. Among these, strain Strep_22 proved to be the most effective, inhibiting almost all tested pathogens. Moreover, this strain protected wheat and tomato roots from soilborne fungal infections and promoted plant growth (Conte et al., 2026). In order to understand the antifungal mechanism showed by Strep_22, in this study genomic and metabolomic analyses have been performed. Moreover, total culture extracts and metabolites more abundant have been used in bio-essays to ascertain their antifungal activity and putative phyto-toxicity against fungal pathogens and young cucumber cotyledons, respectively.

2. Materials and methods

2.1. DNA Extraction and Genome Analysis

Streptomyces strain Strep_22 was purified following the method described by Carlucci et al. (2022). Specifically, culture was purified by spreading them onto water agar (WA) Petri dishes. After overnight incubation at 28 ± 3 °C, single germinating spores or small hyphal fragments were transferred to Petri dishes containing fresh potato dextrose agar (PDA; 39 g/L, Oxoid) in order to

perform molecular identification and characterization. The DNA extraction was conducted according to Carlucci et al. (2013). The quantity and quality of the extracted DNA were assessed using a microvolume spectrophotometer, a microvolume fluorometer, and by electrophoresis on a 1% agarose gel. DNA was sent to Novogene for library preparation and sequencing on an Illumina NovaSeq 6000 platform using a 150bp paired-end approach. Raw reads were pre-processed with Trimmomatic v0.39 (Bolger et al. 2014) (ILLUMINACLIP: TruSeq3-PE-2.fa:2:30:10; SLIDINGWINDOW:5:20; LEADING:5; TRAILING:5; MINLEN:50) in order to remove adapters and low-quality bases. Quality-filtered reads were queried against the comprehensive NCBI core nucleotide (nt) database (released on June 9, 2025; k2_core_nt_20250609) using Kraken2 v2.1.2 (Wood et al. 2019) to assess the potential presence of reads derived from contaminant DNA. Filtered reads were assembled with SPAdes v4.0.0 (Bankevich et al. 2012) (-k 21, 33, 55, 77, 99, 127; --cov-cutoff off; --careful) and polished using Pilon v.1.24 (Walker et al. 2014). Genome assembly statistics were calculated using QUAST v5.2.0 (Gurevich et al. 2013), whereas contamination was assessed with CheckM2 v1.1.0 (Chklovski et al. 2023). Scaffolds with lengths <200 bp were filtered out. Gene prediction and functional annotation were performed with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v2026-04-27.build8516 (Li et al. 2021), using --taxcheck mode to calculate the Average Nucleotide Identity (ANI) (Ciufo et al. 2018). Draft genome visualization, including the identification and representation of Clusters of Orthologous Groups (COGs), was performed using GenoVi v0.4.3 (Cumsille et al. 2023). Antimicrobial resistance (AMR) genes were identified using the Comprehensive Antibiotic Resistance Database (CARD) Resistance Gene Identifier (RGI) v6.0.5 (Alcock et al. 2023). Mobile genetic elements were identified using mobileOG-db v1.1.3 (Brown et al. 2022). Prophages were detected and annotated with PHASTEST v.3.0 (Wishart et al. 2023) and Phigaro v.2.4.0 (Starikova et al. 2020), while the presence of dsDNA and ssDNA viral genomes was assessed using VirSorter v.2.2.4 (Guo et al. 2021). The mlst software v2.33.1 (https://github.com/tseemann/mlst) was used to assign classical bacterial multi-locus sequence types (MLST) and allele profiles using PubMLST-compatible schemes (Jolley et al. 2018). These analyses were performed within Proksee (Grant et al. 2023). Finally, secondary metabolite biosynthetic gene clusters (smBGCs) were predicted with antiSMASH v8.0.8 (Blin et al. 2025) using the 'relaxed' strictness level.

2.2. Metabolite extraction

2.2.1. Culture preparation

The Strep_22 strain inoculum was prepared according to the methodology described by Carlucci et al. (2022). Spores and small mycelium fragments were harvested by scraping the surface of 21-day-

old *Streptomyces* colonies grown on PDA medium at 28.0 ± 3.0 °C in darkness using 2.5 mL of sterilized Tween 20. The suspension was adjusted to a concentration of 10^7 CFU mL^{-1} in a sterile 0.2% Tween 20 solution and then inoculated in 100 mL of liquid GIM (D-glucose 4 g/L; Yeast extract 4 g/L; Malt extract 4 g/L) in a 500 mL flask and incubated at 28.0 ± 3.0 °C and 75 rpm in darkness. After three days, the liquid culture was inoculated in 200 mL of GIM in a 2.0 L flask and incubated at 28.0 ± 3.0 °C and 75 rpm in darkness. After four days, two aliquots of 100 mL of the liquid culture were inoculated in 2.4 L of GIM each in two 5.0 L flask, for a final volume of 5.0 L, and incubated at 28.0 ± 3.0 °C and 100 rpm in darkness.

2.2.2. Crude broth lyophilization

After 28 days, the liquid culture was subjected to sequential filtration, culminating in a final filtration step through a 0.22 µm MF-Millipore ™ filter (Merck, Massachussets, USA). The filtered culture liquid was frozen overnight at -80 °C and subsequently lyophilized for one week using an Alpha 1-4 LSCbasic freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Once completely lyophilized, the cultural broth was conserved at -20°C.

2.2.3. General experimental procedures for chemical analyses

Structural elucidation and purity assessment of all the isolated metabolites were determined by high-resolution mass spectrometry (HRMS) following positive ionization mode, and by comparison of the experimental NMR spectra with spectroscopic data available in literature. NMR spectra were recorded at room temperature on a Bruker spectrometer at 500/125 MHz (Karlsruhe, Germany) spectrometer. Samples were analysed in deuterated chloroform ($CDCl_3$) or metanol (MeOD), using solvent peaks as internal standards. Exact masses were measured by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry, operating in electrospray ionization mode on a Waters SYNAPT G2 high-resolution mass spectrometer (Waters Corporation, Manchester, UK).

Column chromatography (CC) was performed using silica gel (Kieselgel 60, 0.063–0.200 mm, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on analytical and preparative silica gel (Kieselgel 60, F254, 0.25 and 0.5 mm, respectively) or reverse phase (Whatman, KC18, F254, 0.20 mm) (Merck, Darmstadt, Germany) plates. The spots were visualized via exposure to UV light (254 nm) and/or iodine vapours and/or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. Sigma-Aldrich Co. (St. Louis, MO, USA) supplied all the reagents and the solvents.

2.2.4. Extraction and isolation of metabolites

The lyophilized cultural broth was reconstituted in the minimum volume of distilled water, specifically 463 mL. The suspension was extracted using ethyl acetate (EtOAc, 3 × 700 mL), obtaining 382.2 mg. The residue of the production extract was purified by CC eluted with $CHCl_3$/*i*-propanol (9/1, *v/v*) yielding 11 homogeneous fractions (F1-F9). The residue of F2 (156.8 mg) was further purified by CC eluted with $CHCl_3$/*i*-propanol/acetone (from 100/0/0 to 70/15/15, *v/v/v*) yielding eight fractions (2.1-2.8). The residue of 2.2 (43.1 mg) was purified by CC eluted with *n*-hexane/ethyl acetate (5/5, *v/v/v*) yielding eleven fractions (2.2.1-2.2.11). The residues of 2.2.3 (5.8 mg) and 2.2.4 (12.4 mg) were combined and further purified by TLC on direct phase eluted with *n*-hexane/ethyl acetate (6/4, *v/v*) yielding three pure metaboloties (**M1**, 9.1 mg; **M2**, 12.4 mg; **M3**, 4.1 mg). The residue of 2.5 (64.6 mg) was further purified by HPLC yielding to 8 fractions (5.1-5.8). A Phenomenex C18 reversed-phase column Lichrocart (250 × 4.6 mm i.d.; 5 µm) was used. The elution was performed with acetonitrile/H_2O (4/6, *v/v*) 85:15 (formic acid 0.1%) for 12 min. HPLC flow rate was 300 µL/min. The residue 2.5.1 (9.0 mg) was further purified by TLC on direct phase eluted with *n*-hexane/ethyl acetate (6/4, *v/v*) yielding two pure metabolites (**M4**, 1.8 mg; **M5**, 6.5 mg). The residue 2.5.2 (17.3 mg) was further purified by TLC on direct phase eluted with *n*-hexane/ethyl acetate (6/4, *v/v*) yielding a metabolite (**M6**, 2.0 mg).

2.3. Biological assays of culture extracts and metabolites

2.3.1. Antifungal activity

An agar-mycelium disc (4 mm diameter) was taken from the margin of a 21-day-old fungal pathogen grown on PDA and placed face-up on a PDA Petri dish, 0.5 cm from the centre. Subsequently, a 4 mm Whatman No. 2 filter paper disk (Cytiva, Marlborough, Massachusetts, USA) was placed 0.5 cm from the centre of the plate, directly opposite the fungal pathogen disk. Then, the disk was treated with 10 µL of culture extract and metabolite solution. The solution was prepared using 0.5% DMSO. Three different concentrations were tested for each culture extracts and metabolite: C1 (5.0 mg/mL); C2 (3.0 mg/mL); C3 (1.5 mg/mL). The plates were re-sealed and maintained at 22 ± 1 °C in darkness until the fungal pathogens in the control plates reached maximum growth. For slow-growing pathogens, the assay was terminated after 21 days. The experiment was conducted with two replicates for each metabolite-pathogen combination. Control plates consisted of the fungal pathogen inoculated opposite a disk treated with sterile 0.5% DMSO. The inhibitory activity (IA; %) was calculated according to the following formula:

$$IA\ (\%) = \frac{R_1 - R_2}{R_1} \times 100$$

where R_1 was the radial growth of the colony of fungal pathogen towards the edge of the control Petri plate, and R_2 was the radial growth of the colony of fungal pathogen toward the Whatman disk.

2.3.2. Toxicity on Cucumber cotyledons

The putative toxicity of the metabolites was assessed according to Reveglia et al. (2026). Briefly, cotyledons of 7-day-old cucumber (*Cucumis sativus* L.) Marketmore 70 (Semillas Batlle S.A., C/ Pau Vila, 22, 08174 Sant Cugat del Vallès, Barcelona) were gently removed from the plant and surface-sterilized using bleach 0.5% and ethanol 1.0%. First, the cotyledons were placed into a solution of bleach 0.5% for five minutes, and then they were removed from the solution and dipped in a solution of ethanol 1.0% for additionally 5 minutes. Finally, cotyledons were washed with sterile distilled water for 5 minutes and air-dried under laminar airflow in a biosafety cabinet. Disinfected cotyledons were placed face up on soft water agar (Bacteriological agar 5 g/L) and 10 10 µL of the same metabolite solutions used against fungal pathogens were spotted on the first along the main leaf vein. The experiment was conducted with two replicates for each metabolite concentration. Control plates consisted of the cotyledons spotted with sterile DMSO 0.5%. The plates were maintained at 22 ± 1 °C in darkness for seven days. After seven days, the presence of black circle where the solution was spotted, if present.

3. Results

3.1. Genomic identification and characterization

Sequencing yielded 5,434,635 raw reads. Ninety-three percent of these passed quality trimming, and taxonomic assignment confirmed the absence of contamination (Fig. 1A and 1B). Filtered reads were de novo assembled into 210 scaffolds (216 contigs) spanning 7,691,970 bp (55.5% GC content) with an average coverage of 192.271X (N50 = 693,411). There were 51 contigs and 45 scaffolds at least 1,000 bp in length (Table 1). Pilon corrected three SNPs and a single ambiguous base in two contigs, thereby supporting the high accuracy of the assembly. Moreover, the draft genome had 99.99% completeness and 0.06% contamination, as assessed by CheckM2. PGAP predicted 6,707 genes, including 6,612 protein-coding genes and 95 RNA genes. There were 5, 3, and 9 copies of the 5S, 16S, and 23S rRNA genes, respectively, and 75 tRNA genes (Table 1). Sixty percent of the protein-coding genes were associated with at least one Gene Ontology term. The ANI analysis identified strain Strep_22 as *Streptomyces syringium*, with 99.59% ANI and 97.8% query coverage relative to the *S. syringium* type strain genome (GenBank Assembly: GCA_017876625.1; BioSample: SAMN17703988). This value is well above the accepted species delineation threshold of 95-96% (Table 2) (Fig. 2).

AntiSMASH predicted 49 SMGCs in the draft genome of *S. syringium* strain Strep_22 (Fig. 3), suggesting a substantial biosynthetic potential. Among the 49 smBGC, most are related to the production of nonribosomal peptides (NRPS), like antipain and bacillibactin, which are coded in region 1.2 and region 8.2, respectively.

These findings are in line with the COG functional annotation (Fig. 4), which identified numerous genes involved in secondary metabolites biosynthesis, transport, and catabolism (Category Q). Notably, these genes were predominantly organized into discrete genomic regions rather than being evenly distributed throughout the genome, consistent with the organization of biosynthetic gene clusters (Fig. 2).

On the other hand, CARD/RGI analysis identified several putative determinants of antimicrobial resistance (Table 3). Notable examples include HelR, associated with rifamycin resistance, and Rox, involved in rifamycin inactivation. In addition, otr(A) and an EF-Tu variant associated with tetracycline and elfamycin resistance, respectively, were also detected.

3.2. Structural characterization of metabolites

Metabolite **M1** was identified as pimprinine (Fig. 5A) comparison of the experimental ^{1}H and ^{13}C NMR spectra (Fig. 5B and 5C, respectively) with spectroscopic data available in literature for pimprinine (Zhang et al., 2012); and the molecular ion observed by HRMS following positive ionization at 199.0882, calculated 199.0866 for $C_{12}H_{11}N_2O$ $[M+H]^+$.

Metabolite **M2** was identified as pimprinethine (Fig. 6A) comparison of the experimental ^{1}H and ^{13}C NMR spectra (Fig. 6B and 6C, respectively) with spectroscopic data available in literature for pimprinine (Kang et al., 2023); and the molecular ion observed by HRMS following positive ionization at 213.1042, calculated 213.1022 for $C_{13}H_{13}N_2O$ $[M+H]^+$.

Metabolite **M3** was identified as WS-30581A (Fig. 7A) comparison of the experimental ^{1}H and ^{13}C NMR spectra (Fig. 7B and 7C, respectively) with spectroscopic data available in literature for pimprinine (Zhou et al., 2015); and the molecular ion observed by HRMS following positive ionization at 227.1214, calculated 227.1179 for $C_{14}H_{15}N_2O$ $[M+H]^+$.

Metabolite **M4** was identified as cyclo(Pro-Val) (Fig. 8A) comparison of the experimental ^{1}H and ^{13}C NMR spectra (Fig. 8B and 8C, respectively) with spectroscopic data available in literature for pimprinine (Monakhova et al., 2011); and the molecular ion observed by HRMS following positive ionization at 197.1296, calculated 197.1290 for $C_{10}H_{17}N_2O_2$ $[M+H]^+$.

Metabolite **M5** was identified as diethyl phthalate (Fig. 9A) comparison of the experimental ^{1}H and ^{13}C NMR spectra (Fig. 9B and 9C, respectively) with spectroscopic data available in literature for

pimprinine (Ishizu et al., 2022); and the molecular ion observed by HRMS following positive ionization at 245.0795, calculated 245.0790 for $C_{12}H_{14}O_4Na$ $[M+H]^+$.

Metabolite **M6** was identified as cyclo(Phe-Pro) (Fig. 10A) comparison of the experimental ^1H and ^{13}C NMR spectra (Fig. 10B and 10C, respectively) with spectroscopic data available in literature for pimprinine (Ström et al., 2002); and the molecular ion observed by HRMS following positive ionization at 245.1295, calculated 245.1285 for $C_{14}H_{17}N_2O_2$ $[M+H]^+$.

4. Discussion

Strating from the "Green Revolution" chemical synthetic products became relevant for the management of the crops. Their range of application included fertilization and pest management. Agrochemicals allowed farmers to increase the yield of any crops, matching the increasing global demand of food (Devi et al., 2022). In the last decades, especially the most recent ones, both consumers and global politics started worrying about their negative effects, like human illness increase and environment contamination. In facts, it's now known that some chemicals are related to chronic and neurodegenerative illness, useful insect and microorganism death, and general biodiversity loss (Nicolopoulou-Stamati et al., 2016; Oirdi et al., 2024; Xu et al., 2023). The negative impact of chemical pesticides, combined with the concern of the consumers and their increasing interest in healthier products, became the driving force for the application of new laws and Regulations regarding agrochemicals application, especially in the European community (Kudsk and Mathiassen, 2020). The limitation imposed by politics on the application of traditional products promoted the search of new and sustainable strategies. Modern agriculture is relaying on plant-derived products and biological control agents (BCAs) for the management of fungal pathogens, weed, and insects. In facts, these biotic adversities can seriously reduce the yield of the crops, compromising the global food production. Among the BCAs, *Trichoderma* spp. and *Bacillus* spp. are the main BCAs studied and applied by the farmers (Bejarano and Puopolo, 2020). Recently, *Streptomyces* spp. are also becoming fundamental under different aspects. *Streptomyces* are *Actinomycetes* commonly present in the soil, but some species have been isolated also from aquatic environments. They are used not only for the management of fungal pathogens, weed, and insects, but also for promoting the growth of the plants, or even increase the quality of the soil (Wang et al., 2024). *Streptomyces* spp. are the largest natural bio-factory of secondary metabolites, which are reported to have fungicide, herbicide, larvicide, insecticide, and nematocide applications (Alam et al., 2022). Other compounds involved in these processes and produced by *Streptomyces* spp. are enzymes, volatile organic compounds (VOCs), and phytohormones (Jahan et al., 2025). The biological properties of this genus are reflected in the complexity of their genomic. Different species share

similar genome, which is highly related to a common ancestor, which genome was already complex. *Streptomyces* spp. genome resulted composed primarily by secondary metabolite biosynthetic gene clusters (smBGCs), carbohydrate-active enzymes (CAZymes) and protein-coding genes bearing the rare TTA. Specifically, smBGCs are groups of two or more genes involved in the production of secondary metabolites (Abdelsalam et al., 2023; Nikolaidis et al., 2023).

No information is available about *S. syringium* biotic properties, although it seems to be strictly related to *Streptomyces netropsis*. *S. netropsis* is known for the high number of smBGCs, almost 40, and the production of 13 metabolites (Zhang et al., 2021b). This specie is capable to control several fungal pathogens, like *Penicillium italicum*, by producing different metabolites, including pimprinine, amphotericin B, pimprinethine, N-acetylaureothamine, prothracarcin, and aureothin (Yüksel Demirci and Oskay, 2026). Aureothin suppress the growth, reproduction, and behaviour of the insect *Aedes aegypti* and the nematode *Bursaphelenchus xylophilus* (Kang et al., 2022; Kim et al., 2022). Moreover, *S. netropsis* produces phythormones, like auxina and cytokinins, which increase the amount of plant biomass by 1.5-2-fold (Loboda et al., 2024).

Among the 49 smBGC, most are related to the production of nonribosomial peptides (NRPS), like antipain and bacillibactin, which are coded in region 1.2 and region 8.2, respectively.

Plant protection and growth-promotion may be related to the presence of smBGC encoding for nonribosomal peptides (NRPS), which are involved in many processes, like promoting root colonization, modulating root exudates to enhance microbial recruitment and establishment, and inhibiting microorganism growth Guan et al. (2025). For instance, antipain, which has been already reported as a secondary metabolite of *Streptomyces* spp., is a protease inhibitor that blocks DNA repairing process in *Escherichia coli* (Meyn et al., 1977; Suda et al., 1972). Instead, bacillibactin is a *Bacillus* spp. syderophore with three antimicrobial mechanisms of action, including the inhibition of glutamine synthetase, dihydrofolate reductase, and proaerolysin, which are critical bacterial enzymes and considered as the potential antibacterial targets (Prazdova et al., 2025). According to Kumar et al. (2024) significant damage to the hyphal structure of *Alternaria porri* and *Fusarium equiseti* can be observed after treatment with bacillibactin. Moreover, application of 1500 µg/mL bacillibactin on infected tomato leaves demonstrates a substantial inhibition of reactive oxygen speciesROS accumulation, necrosis, and cell death.

Pimprinine (**M1**), pimprinethine (**M2**), and WS-30581 A (**M3**) are indole alkaloids isolated from various *Streptomyces* spp. and have been demonstrated to exhibit broad biotic activities. According to Wei et al. (2014) they are all active against human virus enterovirus 71 (EV71). Pimprinine (**M1**) has been already reported to be a *Streptomyces* spp. metabolite, specifically produced by *Streptomyces pimprina* (Joshi et al., 1936). Pimprinine and its derivates exhibit fungicidal activity against

phytopathogenic fungi, like *Pythium dissimile*, *Alternaria solani*, *Botryotinia fuckeliana* and *Gibberella zeae* (Zhang et al., 2012). The secondary metabolite and its derivate result to be produced also by *Streptomyces netropsis* NZY3 by involving three key enzymes, PimA (GCN5-related *N*-acetyltransferase, GNAT), PimB (pyridoxal 5′-phosphate-dependent tryptophan racemase), and PimC (Fe^{II}/α-ketoglutarate-dependent dioxygenase, Fe^{II}/αKGD) (Zhang et al., 2025).

According to Tejaswi et al. (2026), pimprinethine (**M2**) produced by *S. netropsis* IICTRSP-174 has high therapeutic potential. Pimprinethine produced by *S. netropsis* NBRC 3723 shows nematocide properties against *Bursaphelenchus xylophilus*, killing the total population within 24 hours reported (Kang et al., 2023). Pimprinethine and its derivates are anticancer, pesticidal, antimicrobial, antiviral, antioxidant, herbicidal, insecticidal. and anti-inflammatory agents (Liu et al., 2024; Zhang et al., 2021a). According to Liu et al. (2022), pimprinethine displays high inhibition against *G. zeae* and Alternaria Leaf Spout.

WS-30581A (**M3**) is also reported to be a *Streptomyces* spp. metabolite, in fact it's produce by *Streptomyces* sp. WS-13317 (Wei et al., 2014). Although there is no information about its application in agriculture, its derivates exhibit aintifungal activity against *G. zeae* and Alternaria Leaf Spout (Liu et al., 2022)

According to Chen et al. (2018) cyclo(Pro-Val) (**M4**) produced by *Streptomyces nigra* shows high antitumoral activity in *in vitro* condition. The compound seems to be able to inhibit the production of aflatoxine by *Aspergillus parasiticus* repressing the transcription of the related genus *aflR*, *hexB*, *pksL1*, and *dmtA* (Yan et al., 2004).

Diethyl phthalate (**M5**) is also reported to be a secondary metabolite produced by *Streptomyces* spp. According to Rammali et al. (2024), diethyl phthalate is the main secondary metabolite produced by *Streptomyces* sp. EIZ2, which shows high inhibition against *Pseudomonas aeruginosa* ATCC 27,853, *S. aureus* ATCC 25,923, *E. coli* ATCC 25,922, and *Candida albicans* ATTC 60193. Diethyl phthalate produced by *Streptomyces albus* DR57 exhibits antibacterial, anti-inflammatory, and anticancer activities (Devi et al., 2025). Kumari and Rao (2025) identified diethyl phthalate as the key compound responsible for cysteine protease inhibition. Cysteine protease is involved in protein degradation by cleaving peptide bonds.

Cyclo(Phe-Pro) (**M6**) also is reported to be a *Streptomyces* spp. secondary metabolite. Indeed, according to Li et al (2006), this metabolite is produced by *Streptomyces fungicidicus*, a *Streptomyces* sp. isolated form the Pacific sediment, and it shows antilarval activity against *Balanus amphitrite*. In addition, cyclo(Phe-Pro) produced by *Lactobacillus plantarum* MiLAB 393 inhibits *Fusarium sporotrichioides* and *Aspergillus fumigatus* (Ström et al., 2002).

Regarding the results obtained in this study, *Streptomyces* sp. strain Strep_22, identified as *Streptomyces syringium*, may be considered a valid alternative for the management of soilborne fungal pathogens due to its inhibitory activities against fungal pathogens affecting wheat and tomato crops (Conte et al., 2026). Although no information is available on the biological properties of this species, the closely related *Streptomyces netropsis* is also known for its efficacy in controlling fungal pathogens. The high inhibitory activity showed by *S. syringium* seems to be strongly related to the production of secondary metabolites, such as cyclo(Phe-Pro), cyclo(Pro-Val), diethyl phthalate, pimprinine, pimprinethine, and WS-30581 A as they were the main metabolites produced and characterized. The latter metabolites have been reported to have a wide range of application, from pest management in open field to human health, but none of them have been reported to promote the growth of the plants. Regarding the biological activities performed by bio-essays, the results proved disappointing due to the small sample sizes of culture and metabolites available. Therefore, this aspect of study warrants further investigation, regarding both the extraction methodologies and the evaluation of fungal antagonism and phyto-toxicity activities. Since, in this study *S. syringium* (strain Strep_22) also showed plant-growth promoting effects, other un-identified metabolites here extracted deserve further to be characterised and essay their biological activities. Thus, the next step of this study are to keep on isolate and characterise the secondary metabolites produced by Strep_22, and to test and confirm its efficacy in open field.

5. Conclusions

In conclusion, the urgent shift toward sustainable agriculture away from harmful chemical agrochemicals highlights the immense biocontrol potential of *Streptomyces* spp. This study demonstrates that *Streptomyces syringium* (strain Strep_22), a species previously un-characterized for its biological properties, serves as a highly promising alternative for managing destructive soilborne fungal pathogens in wheat and tomato crops, while simultaneously acting as a plant-growth promoter. Its strong inhibitory efficacy is largely driven by a rich secondary metabolome, characterized by key bioactive compounds such as cyclo(Phe-Pro), cyclo(Pro-Val), diethyl phthalate, pimprinine, pimprinethine, and WS-30581 A. However, due to limited culture and metabolite sample sizes, the preliminary bio-assays yielded inconclusive results regarding antagonism and phytotoxicity. Consequently, further investigation is strongly warranted to optimize extraction methodologies, re-evaluate these specific biological activities, and fully characterize the remaining un-identified metabolites responsible for the observed plant-growth-promoting effects. Ultimately, the necessary next steps for this research must focus on continuing the isolation of Strep_22 secondary metabolites and conducting extensive open-field trials to validate and confirm its practical, real-world efficacy.

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Table 1. Genome assembly statistics of *Streptomyces syringium* strain Strep_22.

	Contigs	Scaffolds	Summary of gene prediction	
# contigs (>= 0 bp)	216	210	Genes (total)	6,707
# contigs (>= 1000 bp)	51	45	CDS (total)	6,612
# contigs (>= 5000 bp)	38	36	Genes (coding)	6,514
# contigs (>= 10000 bp)	34	31	CDSs (with protein)	6,514
# contigs (>= 25000 bp)	29	26	Genes (RNA)	95
# contigs (>= 50000 bp)	21	18	rRNAs	5, 3, 9 (5S, 16S, 23S)
Total length (>= 0 bp)	7691730	7691970	Complete rRNAs	5, 1, 1 (5S, 16S, 23S)
Total length (>= 1000 bp)	7635917	7636157	Partial rRNAs	2, 8 (16S, 23S)
Total length (>= 5000 bp)	7604761	7615010	tRNAs	75
Total length (>= 10000 bp)	7567938	7572773		
Total length (>= 25000 bp)	7483383	7488218		
Total length (>= 50000 bp)	7201126	7205961		
# contigs	51	45		
Largest contig	763932	1038817		
Total length	7635917	7636157		
GC (%)	71.71	71.71		
N50	430174	693411		
N90	99812	181357		
auN	450369.2	576708.4		
L50	7	5		
L90	17	14		
# N's per 100 kbp	0.00	2.97		

All statistics are based on contigs of size >= 1000 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs).

Table 2. Average nucleotide identity (ANI) analysis on the assembled genome.

ANI	Coverages	NewSeq	CntmSeq	CntmSeqANI	Assembly	Flg	Organism (assembly_accession, assembly_name)
99.592	(97.8 97.4)	7512787	7512787	100	26150438		*Streptomyces syringium* (GCA_017876625.1, ASM1787662v1)
93.776	(81.7 77.2)	17074	17074	92	55312598		*Streptomyces netropsis* (GCA_037414505.1, Str_netropsis_B-235_v1)
93.772	(82.3 78.7)	4867	4867	88	21607868		*Streptomyces netropsis* (GCA_014203545.1, ASM1420354v1)
93.844	(80.1 79.7)	0	0	0	22192038		*Streptomyces netropsis* (GCA_014648835.1, ASM1464883v1)
85.544	(50.7 50.3)	665	665	88	58707628		*Streptomyces stramineus* (GCA_039524935.1, ASM3952493v1)
85.391	(50.8 49.9)	106	106	96	62891168		*Streptomyces stramineus* (GCA_042674825.1, ASM4267482v1)
85.354	(38.7 43.4)	0	0	0	1154938		*Streptomyces lilacinus* (GCA_000715685.1, ASM71568v1)
85.103	(47.7 46.3)	659	659	85	5903578		*Streptomyces eurocidicus* (GCA_002891295.1, ASM289129v1)
85.214	(47.9 43.6)	0	0	0	4990528		*Streptomyces albireticuli* (GCA_002286695.1, ASM228669v1)
85.104	(47.6 46.4)	0	0	0	21607828		*Streptomyces eurocidicus* (GCA_014203505.1, ASM1420350v1)
85.121	(47.6 46.3)	0	0	0	23190758		*Streptomyces eurocidicus* (GCA_015475845.1, ASM1547584v1)
85.036	(47.7 45.3)	1343	1343	81	58703858		*Streptomyces blastmyceticus* (GCA_039521185.1, ASM3952118v1)
85.047	(48.8 43.8)	1013	1013	84	30640888		*Streptomyces albireticuli* (GCA_021228125.1, ASM2122812v1)
84.833	(45.3 42.4)	0	0	0	18403428		*Streptomyces telluris* (GCA_012034305.1, ASM1203430v1)
84.568	(46.9 50.6)	944	944	87	22193698		*Streptomyces cinnamoneus* (GCA_014650495.1, ASM1465049v1)
84.627	(47.9 42.6)	0	0	0	2687768		*Streptomyces roseifaciens* (GCA_001445655.1, ASM144565v1)
84.587	(43.0 59.7)	0	0	0	21607888		*Streptomyces olivoverticillatus* (GCA_014203555.1, ASM1420355v1)
84.582	(46.3 45.9)	0	0	0	22193538		*Streptomyces hiroshimensis* (GCA_014650335.1, ASM1465033v1)
84.644	(48.8 50.9)	0	0	0	22198798		*Streptomyces griseocarneus* (GCA_014655595.1, ASM1465559v1)

ANI: ANI value between this assembly and the type listed in this row; (Coverages) : query-coverage and subject-coverage of this assembly (query) and the type (subject); NewSeq: the count of bases best assigned to this type assembly; CntmSeq: the portion of NewSeq allocated for purposes of evaluating contamination; CntmSeqANI: ANI value of the CntmSeq portion against the type; Flg: Type flags; currently: C = contaminant; E = effectively published; T = trusted species: Assembly: Release-id of the type-assembly (this value matches the accession and assembly-name on the right column); Organism: Organism of this type-assembly; (assembly_accession, assembly_name): of this type-assembly.

Table 3. Prediction of antibiotic resistance genes using the Resistance Gene Identifier (RGI) and the Comprehensive Antibiotic Resistance Database (CARD) in the draft genome of *Streptomyces syringium* strain Strep_22.

Best_Hit_ARO	Best_Identities (%)	Model_type	Drug Class	Resistance Mechanism	AMR Gene Family	Percentage Length of Reference Sequence	ID	Antibiotic
qacG	40.19	protein homolog model	disinfecting agents and antiseptics	antibiotic efflux	small multidrug resistance (SMR) antibiotic efflux pump	100.00	gnl|BL_ORD_ID|4668|hsp_num:0	benzalkonium chloride
Streptomyces venezuelae rox	69.12	protein homolog model	rifamycin antibiotic	antibiotic inactivation	rifampin monooxygenase	103.36	gnl|BL_ORD_ID|4848|hsp_num:0	rifampin; rifaximin; rifapentine
Streptomyces lividans otr(A)	60.41	protein homolog model	tetracycline antibiotic	antibiotic target protection	tetracycline-resistant ribosomal protection protein	103.29	gnl|BL_ORD_ID|4822|hsp_num:0	tetracycline
vanT gene in vanG cluster	31.54	protein homolog model	glycopeptide antibiotic	antibiotic target alteration	glycopeptide resistance gene cluster; vanT	54.63	gnl|BL_ORD_ID|1516|hsp_num:0	vancomycin
HelR	78.09	protein homolog model	rifamycin antibiotic	antibiotic target protection	helicase-like RNA polymerase protection protein	99.31	gnl|BL_ORD_ID|4852|hsp_num:0	rifampin; rifaximin; rifabutin
Streptomyces cinnamoneus EF-Tu mutants conferring resistance to elfamycin	99.75	protein variant model	elfamycin antibiotic	antibiotic target alteration	elfamycin resistant EF-Tu	100.00	gnl|BL_ORD_ID|2080|hsp_num:0	kirromycin

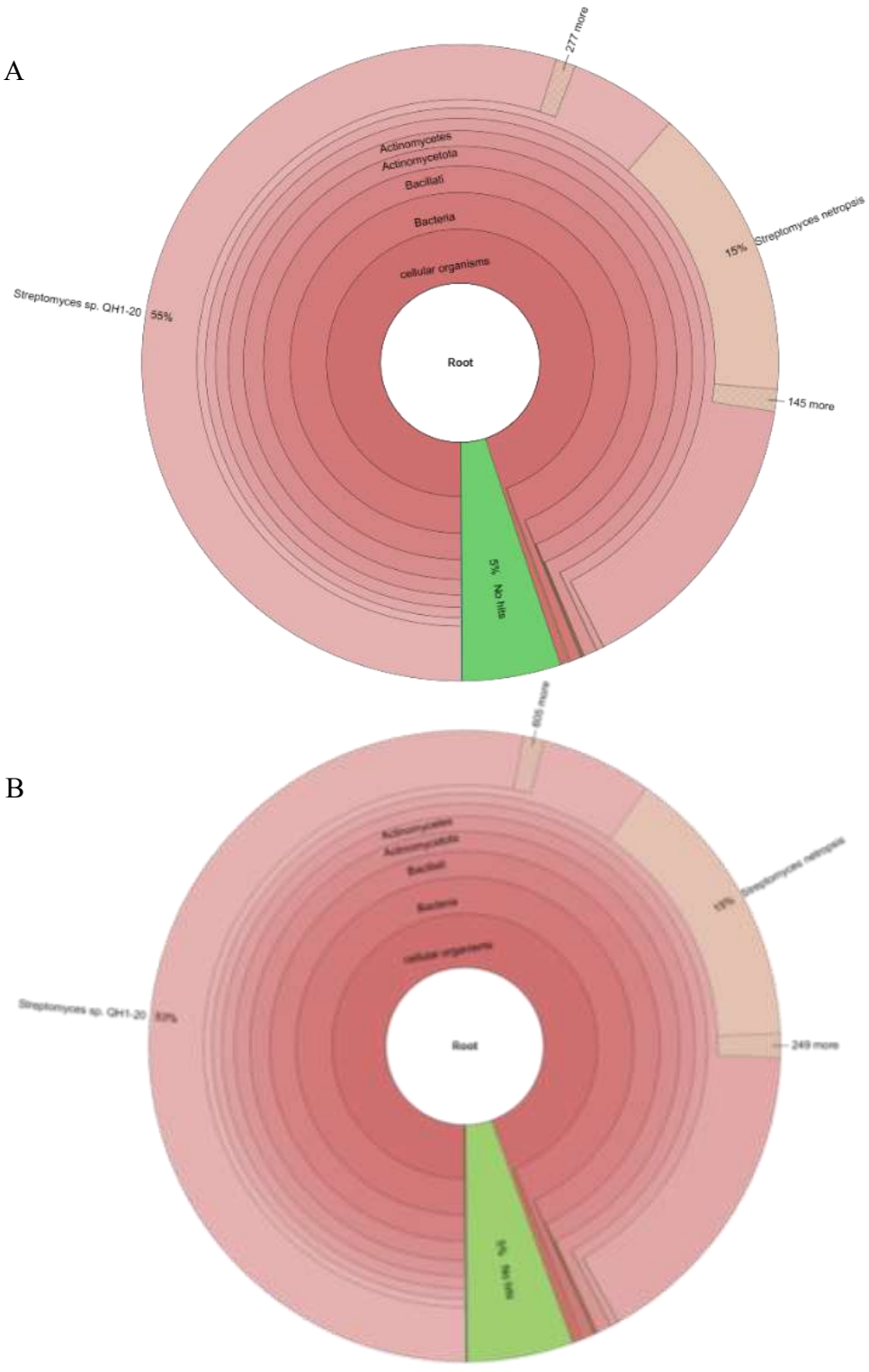


Fig. 1. Krona plot showing the taxonomic classification of quality-filtered reads obtained from the analysis of Kraken2 v2.1.2 against the NCBI core nucleotide (nt) database (k2_core_nt_20250609). Panels represent different sequencing libraries.

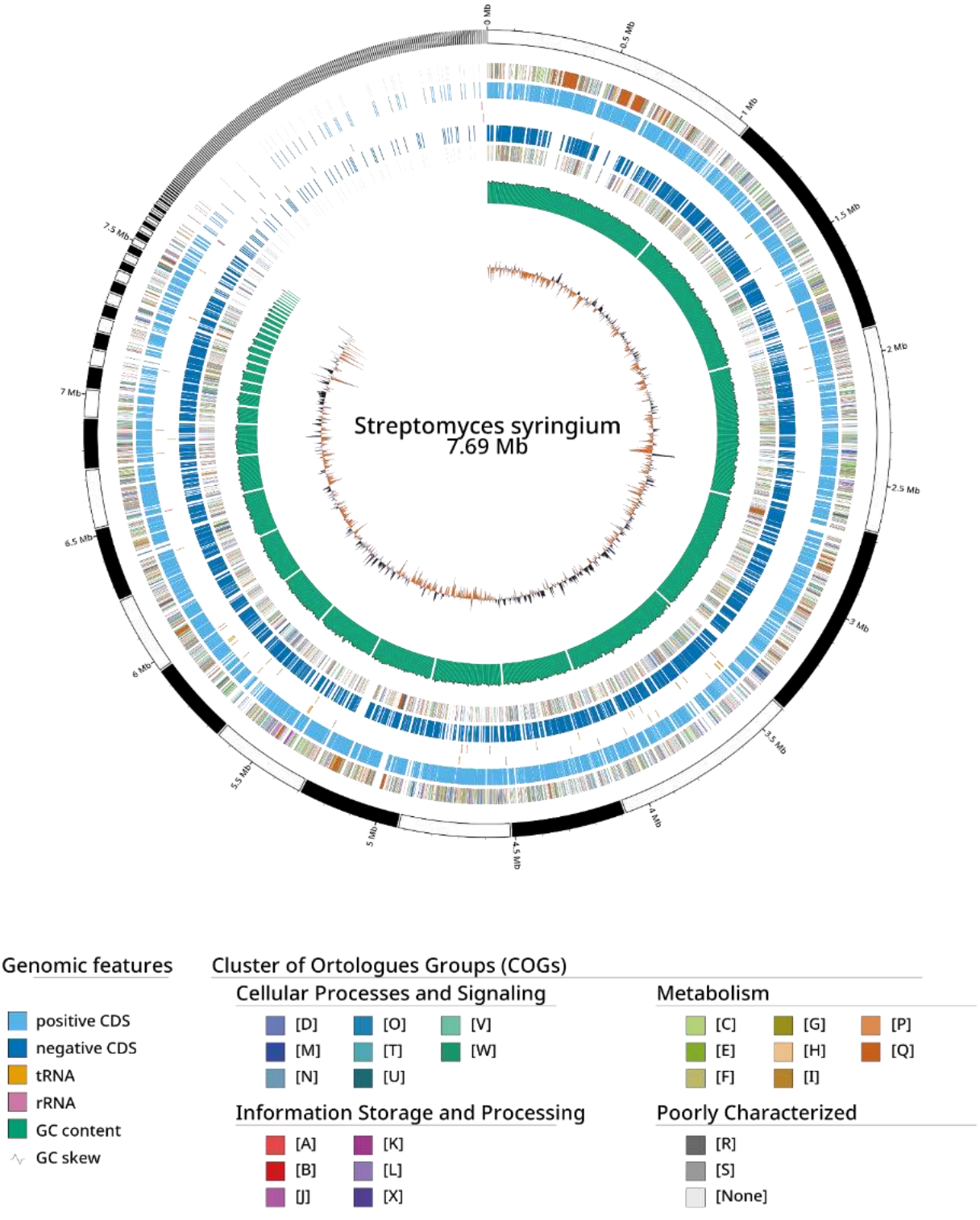


Fig. 2. Circular map of the draft genome of *Streptomyces syringium* strainStrep_22. Each contig is represented as a separate band on a circular representation.

Region	Type	From	To	Similarity Confidence	Most similar known cluster	
Region 1.1	T1PKS , PKS-like , NRPS , transAT-PKS , terpene	247,107	437,877	Medium	kirromycin	NRPS:Type I+PKS:Type I
Region 1.2	NRPS-like , NRPS	454,934	503,225	High	antipain	NRPS:Type I
Region 1.3	T1PKS , terpene , RiPP-like , azole-containing-RiPP , lanthipeptide-class-i , NRPS-like , NRPS	531,359	823,937	Medium	nystatin A1	PKS:Type I+saccharide:hybrid/tailoring
Region 1.4	hglE-KS	854,309	900,407			
Region 1.5	terpene	942,995	978,334			
Region 1.6	hglE-KS	991,652	1,037,768			
Region 3.1	melanin	75,786	86,202			
Region 3.2	linaridin	166,934	187,512			
Region 3.3	lanthipeptide-class-iii	213,942	236,623	High	SapB	ribosomal:RiPP:Lanthipeptide
Region 3.4	RiPP-like	287,660	298,838			
Region 3.5	ectoine	669,724	680,122	High	ectoine	other:other
Region 3.6	terpene	711,089	732,240			
Region 4.1	NRPS , T1PKS , terpene	6,344	83,354	High	althiomycin	NRPS:Type I+PKS:Type I
Region 4.2	terpene-precursor , RiPP-like , NRPS	100,841	214,057	High	leupeptin Pr/leupeptin Ac	NRPS:Type I
Region 4.3	aminopolycarboxylic-acid	252,019	265,508	High	Ethylenediaminesuccinic acid hydroxyarginine	other:other
Region 4.4	T3PKS	281,238	322,293	Low	violapyrone B	PKS
Region 4.5	T1PKS	354,242	400,040	Medium	uncialamycin	PKS
Region 4.6	other	400,749	442,104			
Region 4.7	NI-siderophore	483,214	513,004	Medium	legonoxamine A/desferrioxamine B/legonoxamine B	other:other
Region 4.8	T1PKS , lanthipeptide-class-iv	520,197	567,723			
Region 4.9	terpene	629,620	651,677	Low	sarasinoside	other:other
Region 5.1	butyrolactone	134,599	145,663			
Region 5.2	lanthipeptide-class-v	355,905	397,985	High	pristinin A3	ribosomal:RiPP
Region 5.3	HR-T2PKS , T3PKS	557,793	615,218	Low	vazabitide A	NRPS:Type I
Region 5.4	T3PKS	623,009	664,184	Low	loseolamycin A1/loseolamycin A2	PKS
Region 6.1	T1PKS	230,551	276,985			
Region 8.1	NRPS-like , arylpolyene , NRPS , terpene-precursor , T1PKS	44,177	182,123	Medium	kitacinnamycin A/kitacinnamycin B/kitacinnamycin C/kitacinnamycin D/kitacinnamycin E/kitacinnamycin F	NRPS:Type I
Region 8.2	NRPS-like , NRP-metallophore , NRPS	261,614	339,385	High	bacillibactin	NRPS:Type I
Region 8.3	butyrolactone	351,842	362,612			
Region 9.1	T1PKS	155,938	206,053			
Region 10.1	melanin	113,560	123,976	Low	melanin	other:other
Region 10.2	terpene	251,272	273,236			
Region 11.1	terpene , T1PKS , NRPS , T2PKS	34,394	138,550	Medium	JBIR-76/JBIR-77	PKS
Region 12.1	terpene	142,272	163,315			
Region 12.2	NI-siderophore	165,158	197,941	Low	peucechelin	NRPS:Type I
Region 13.1	NI-siderophore	41,441	71,390	Low	kinamycin	PKS
Region 14.1	RRE-containing	171,110	181,357	Low	lagmysin	ribosomal:RiPP
Region 15.1	hydrogen-cyanide	56,534	69,523	Low	aborycin	ribosomal:RiPP
Region 15.2	RRE-containing	88,967	99,812	Low	bottromycin A2	ribosomal:RiPP:Bottromycin
Region 18.1	terpene	1	19,662	Medium	hopene	terpene
Region 20.1	RRE-containing	30,818	41,084			
Region 21.1	NRPS	10,589	39,914			
Region 22.1	indole	1	16,310			
Region 23.1	NRPS-like , lassopeptide	1	25,201	High	citrulassin B	ribosomal:RiPP
Region 24.1	T3PKS	1	27,118	High	2-methoxy-5-methyl-6-(13-methyltetradecyl)-1,4-benzoquinone/2-methoxy-5-methyl-6-(13-methyltetradecyl)phenol	PKS:Type III
Region 26.1	T1PKS	1	25,213			
Region 29.1	terpene	4,812	16,523			
Region 32.1	terpene	1	9,369			
Region 35.1	RiPP-like , bottromycin	1	8,847	Medium	bottromycin A2	ribosomal:RiPP:Bottromycin

Fig. 3. Summary of the secondary metabolite gene clusters (SMGCs) predicted by antiSMASH v8.0.8 in the draft genome of *Streptomyces syringium* strain Strep_22. The most similar known clusters in the curated MIBiG database are also shown.

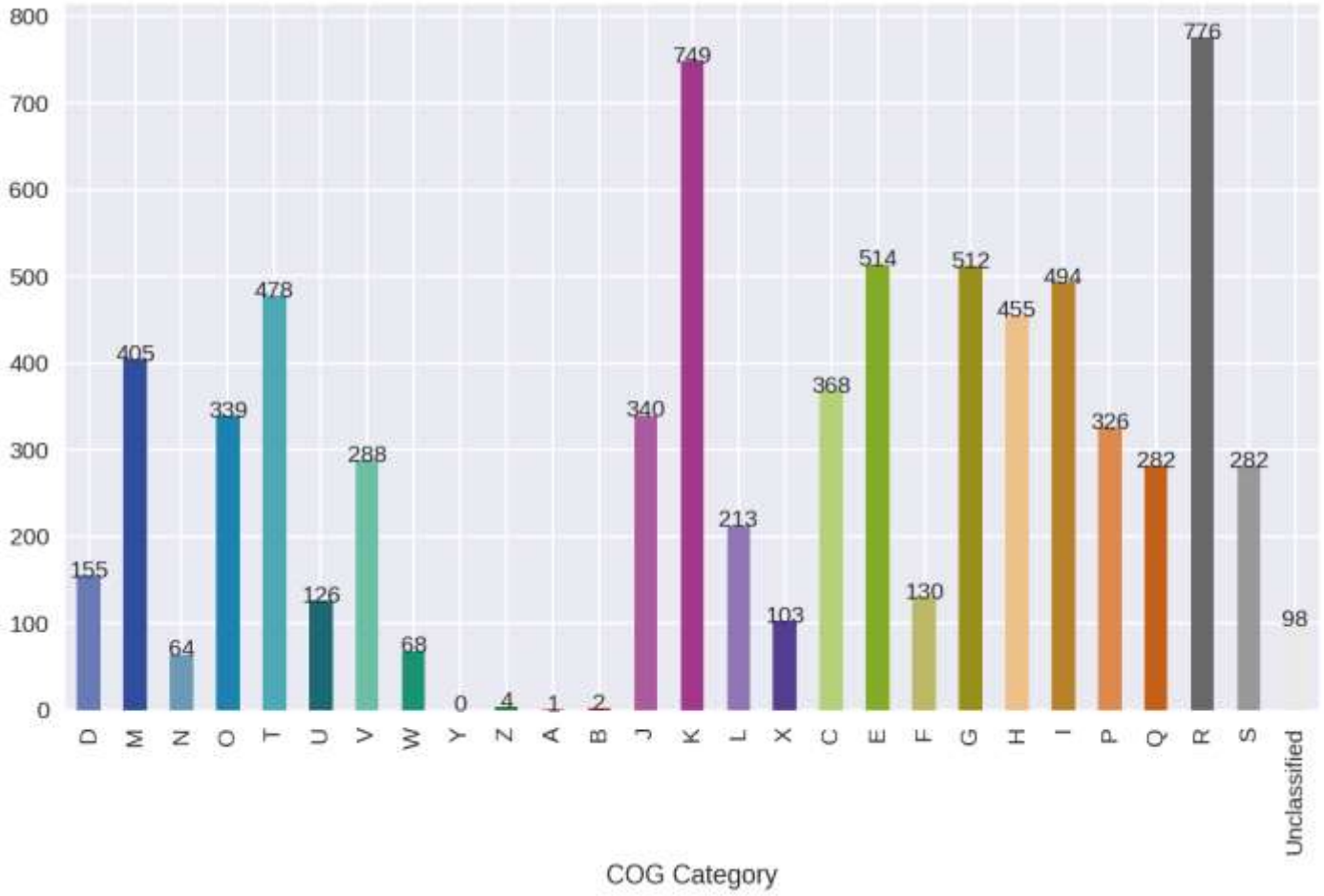


Fig. 4. Histogram showing the abundance of Cluster of Orthologous Groups (COG) categories in the draft genome of *Streptomyces syringium* strain Strep_22. COG categories: [J] Translation, ribosomal structure and biogenesis, [A] RNA processing and modification, [K] Transcription, [L] Replication, recombination and repair, [B] Chromatin structure and dynamics, [D] Cell cycle control, cell division, chromosome partitioning, [V] Defence mechanisms, [T] Signal transduction mechanisms, [M] Cell wall/membrane/envelope biogenesis, [N] Cell motility, [Z] Cytoskeleton, [W] Extracellular structures, [U] Intracellular trafficking, secretion, and vesicular transport, [O] Posttranslational modification, protein turnover, chaperones, [C] Energy production and conversion, [G] Carbohydrate transport and metabolism, [E] Amino acid transport and metabolism, [F] Nucleotide transport and metabolism, [H] Coenzyme transport and metabolism, [I] Lipid transport and metabolism, [P] Inorganic ion transport and metabolism, [Q] Secondary metabolites biosynthesis, transport and catabolism, [R] General function prediction only, [S] Function unknown.

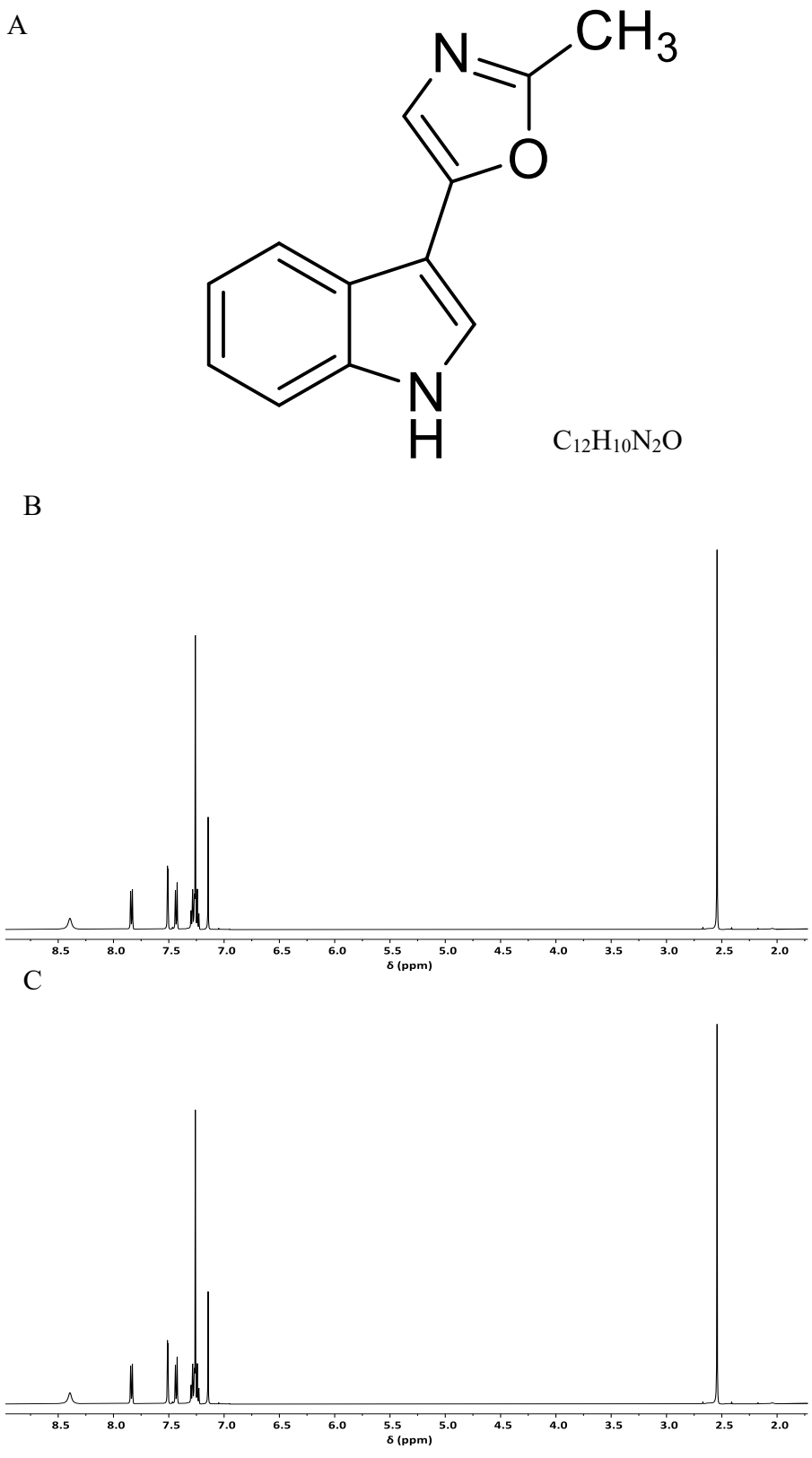


Fig. 5. Chemical characteristics of pimprinine (**M1**). A: Structure of pimprinine; B: ^{1}H NMR spectrum of pimprinine recorded in $CDCl_3$ at 500 MHz; C: ^{13}C NMR spectrum of pimprinine recorded in $CDCl_3$ at 125 MHz.

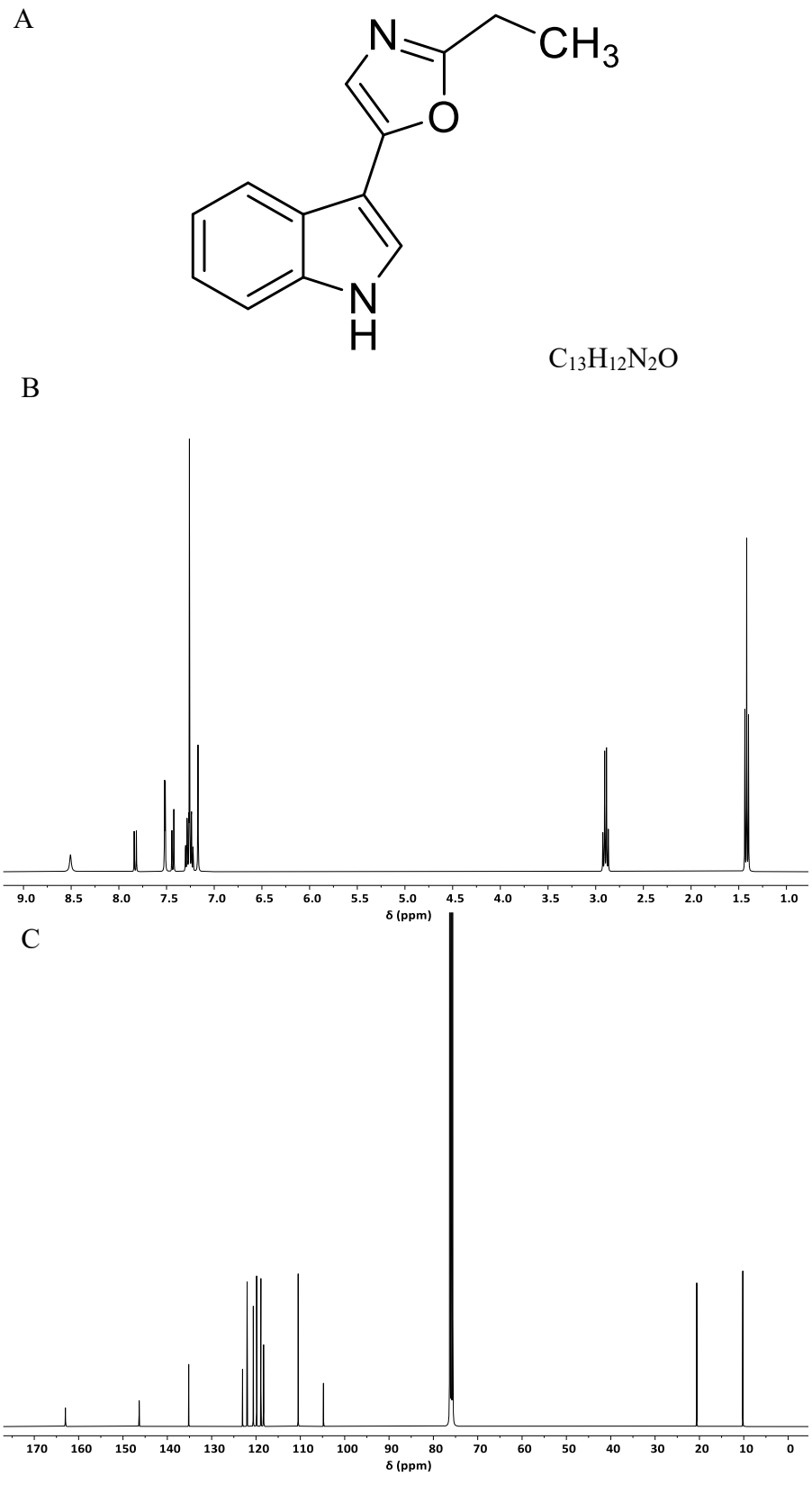


Fig. 6. Chemical characteristics of pimprinethine (**M2**). A: Structure of pimprinethine; B: ^{1}H NMR spectrum of pimprinethine recorded in $CDCl_3$ at 500 MHz; C: ^{13}C NMR spectrum of pimprinethine recorded in $CDCl_3$ at 125 MHz.

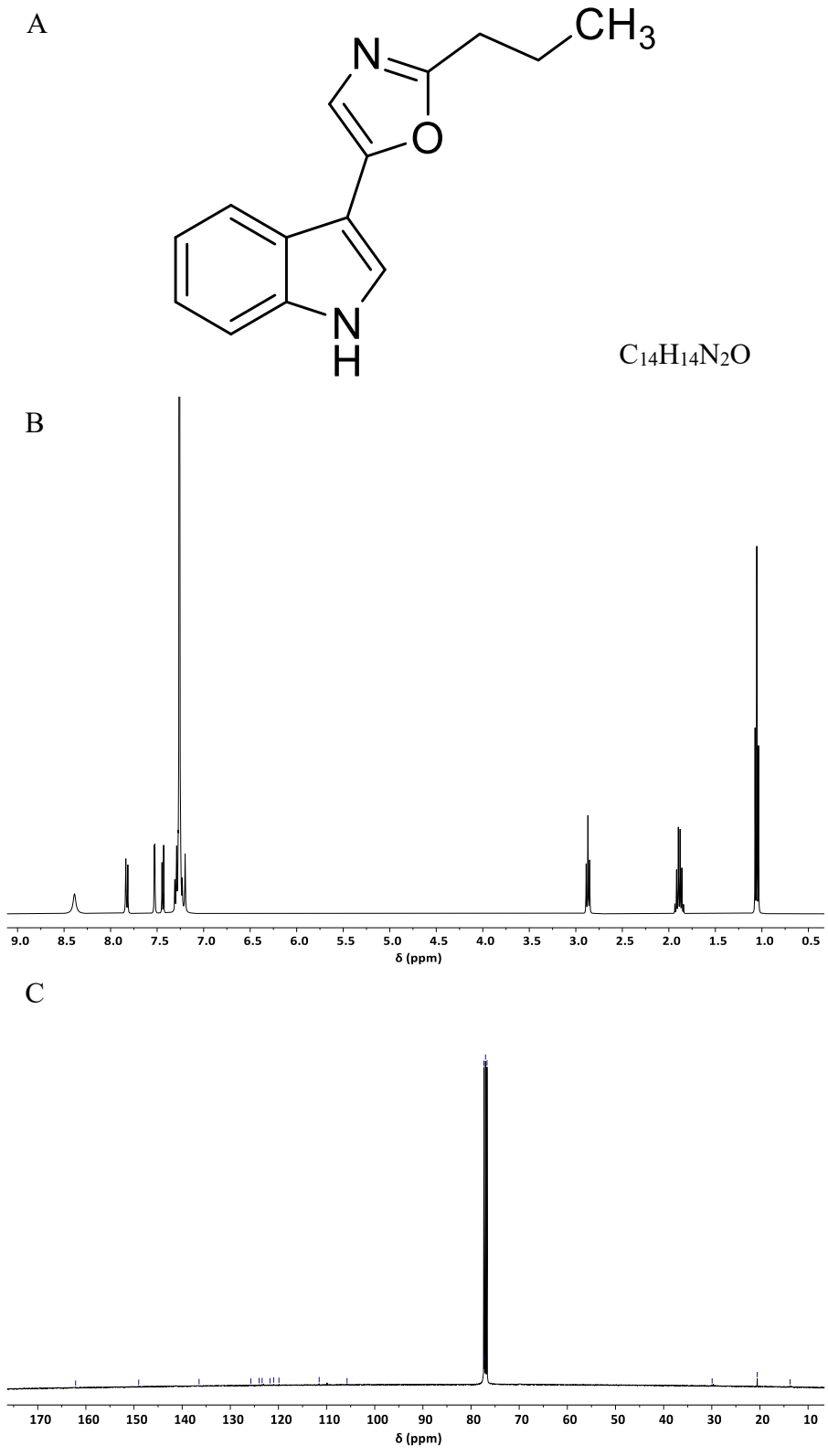
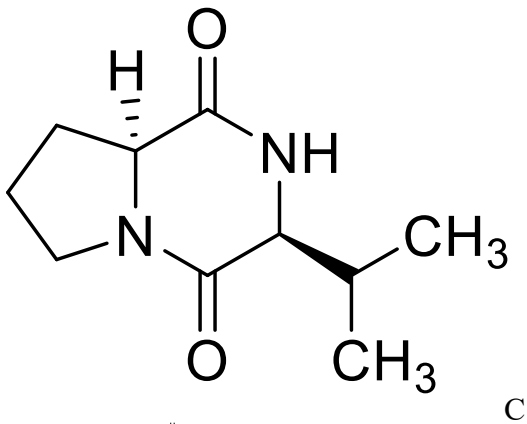


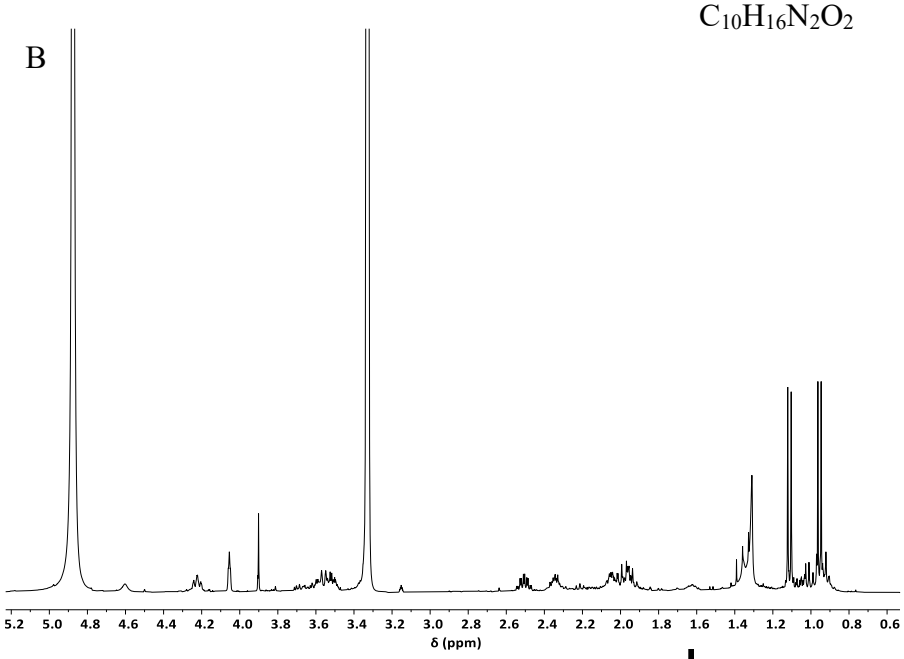
Fig. 7. Chemical characteristics of WS-30581A (**M3**). A: Structure of WS-30581A; B: ^{1}H NMR spectrum of WS-30581A recorded in $CDCl_3$ at 500 MHz; C: ^{13}C NMR spectrum of WS-30581A recorded in $CDCl_3$ at 125 MHz.

A



$C_{10}H_{16}N_2O_2$

B



C

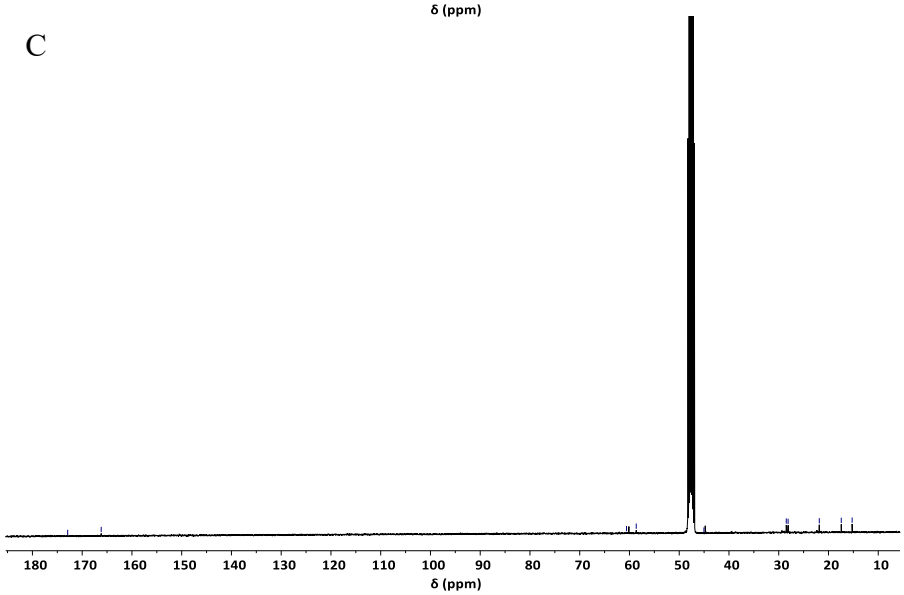


Fig. 8. Chemical characteristics of cyclo (Pro-Val) (**M4**). A: Structure of cyclo (Pro-Val); B: ^{1}H NMR spectrum of cyclo (Pro-Val) recorded in MeOD at 500 MHz; C: ^{13}C NMR spectrum of cyclo (Pro-Val) recorded in MeOD at 125 MHz.

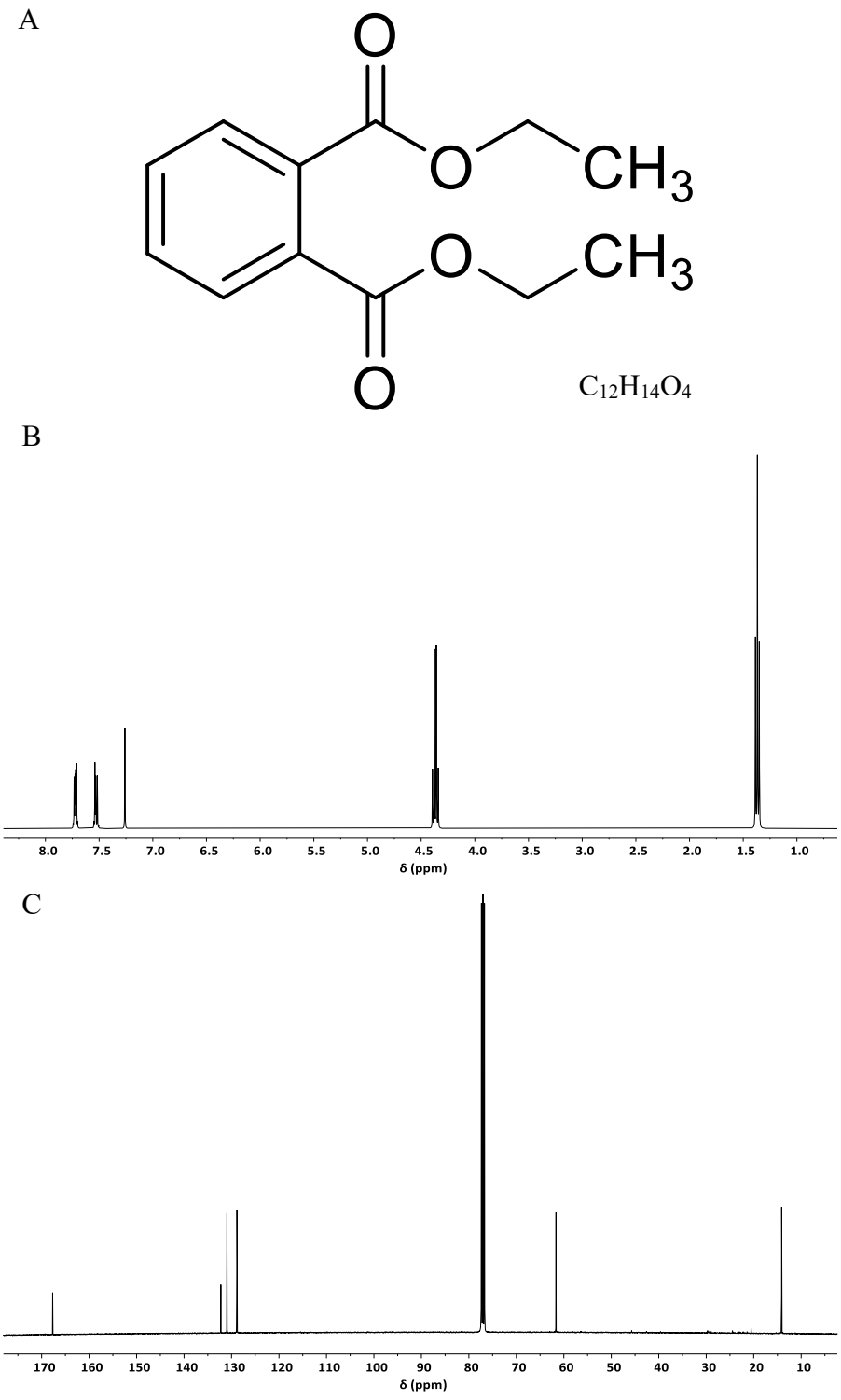


Fig. 9. Chemical characteristics of diethyl phthalate (**M5**). A: Structure of diethyl phthalate; B: ^{1}H NMR spectrum of diethyl phthalate recorded in $CDCl_3$ at 500 MHz; C: ^{13}C NMR spectrum of diethyl phthalate recorded in $CDCl_3$ at 125 MHz.

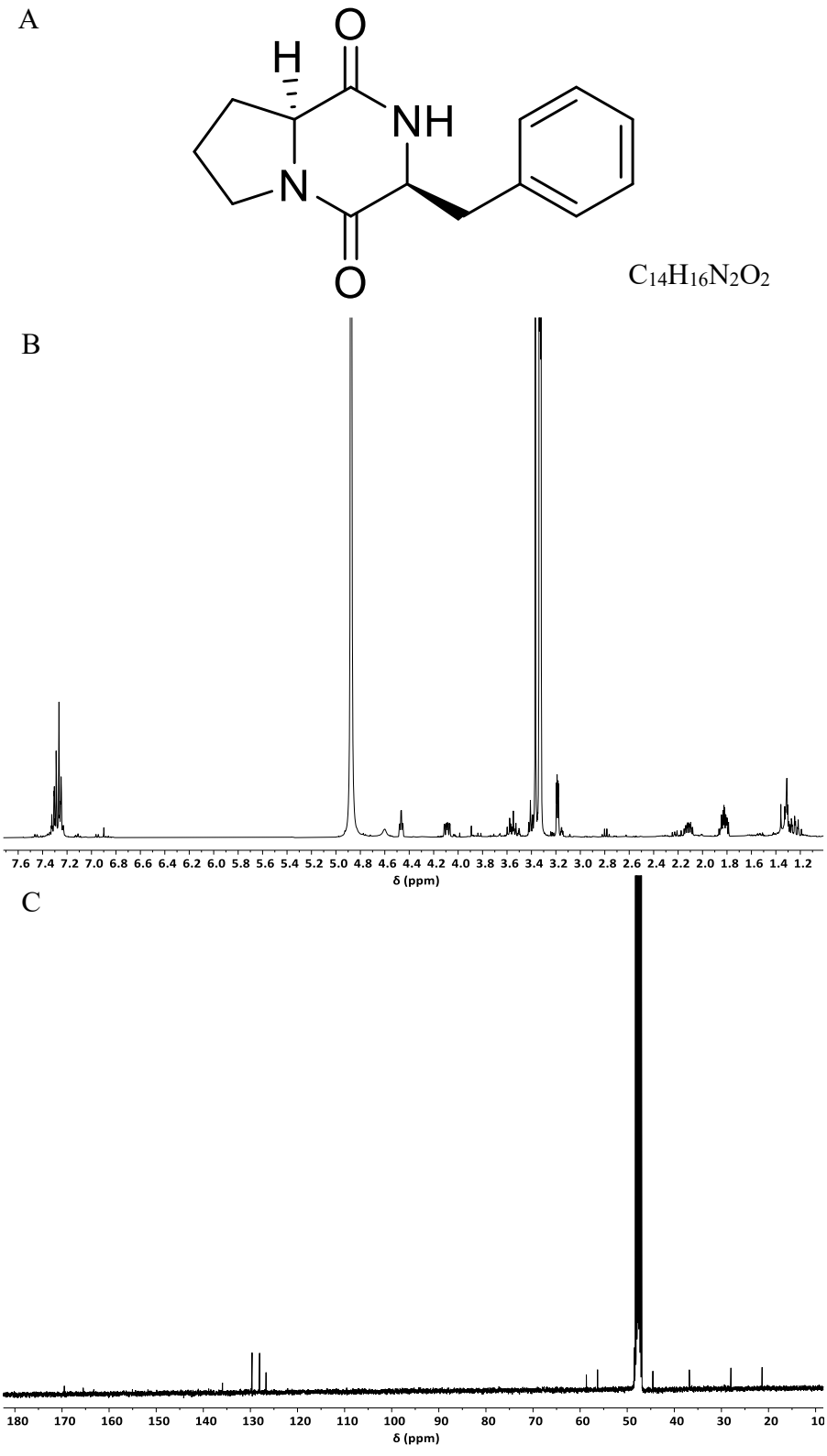


Fig. 10. Chemical characteristics of cyclo (Phe-Pro) (**M6**). A: Structure of cyclo (Phe-Pro); B: ^{1}H NMR spectrum of cyclo (Phe-Pro) recorded in MeOD at 500 MHz; C: ^{13}C NMR spectrum of cyclo (Phe-Pro) recorded in MeOD at 125 MHz.

PART III

3. GENERAL CONCLUSIONS

Powders obtained from agricultural wastes represent a valid alternative for the management of the fungal phytopathogens. Powders obtained from eucalyptus and tomato leaves, bergamot and pomegranate peel, and commercial organic powdery products, like EP5, are able to control partially or totally the growth of fungal pathogens in *in vitro* condition. Specifically, EP5 inhibits 29 of the 31 tested fungal pathogens, while bergamot, eucalyptus, pomegranate, and tomato inhibit the 68% of the tested fungal pathogens. Bergamot, eucalyptus, pomegranate, and EP5 result to be effective also against soilborne fungal pathogens of wheat in *in vitro* and *in vivo* conditions. Specifically, regarding the *in vivo* experiment, high doses of bergamot, pomegranate, and EP5 show high inhibition of the growth of *Fusarium algeriense*, *Fusarium culmorum*, and *Fusarium oxysporum* f.sp. *dianthi*, but also high phytotoxicity on the roots and growth of wheat. Interestingly, the powders are suitable for biofumigation. In fact, the powders maintaine their efficacy on the three *Fusarium* spp. over time, but not the phytotoxic effect on the plants. Re-sowing kernels in the soil previously artificially inoculated and treated shows a normal growth, but the powders remain efficient against the fungal pathogens. Interestingly, EP5 shifts from being highly phytotoxic to promote the growth of the wheat plants. The three powders show a similar effect on the weed of wheat in *in vitro* and *in* vivo conditions. Their water extracts successfully inhibit the germination of *Alopecurus myosuroides* and *Lolium multiflorum* in *in vitro* conditions. The phythotoxic/residual effect of bergamot, pomegranate, and EP5 can be observed also on weeds, suggesting that they are suitable not only for the sustainable management of the fungal pathogens of wheat, but also for the sustainable management of its associated weeds. In fact, the powders inhibit also the growth of *A. myosuroides* and *L. multiflorum* in *in vivo* conditions, but also the germination of kernels and growth of wheat. Also in this case, the powders control weeds over time, without negatively affecting the growth of wheat. This outcome suggests that the powders can be applied against weeds emerged before the sowing of kernels, or sprayed directly on grown weed as water extracts. To avoid their phytotoxic effect, it is possible to apply low doses of bergamot, pomegranate, and EP5. Indeed, low doses of bergamot, pomegranate, and EP5 show no phytotoxicity on tomato roots, but are still capable to inhibit the growth and infection of *Agroathelia rolfsii*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*. Similarly, the treatment with the powders does not affect any of the growth parameters of tomato, but successfully controls the germination and growth of *Amaranthus retroflexus*, *Chenopodium album*, and *Solanum nigrum*. Thus, bergamot, pomegranate, and EP5 are suitable for the sustainable management of soilborne fungal pathogens and weed of tomato. Apart from the plant-derived product obtained from the agricultural wastes, another suitable means for the sustainable management of fungal pathogens

are *Streptomyces* spp., especially due to the wide range of secondary metabolites that they produce. Three tested strains, Strep_22, Strep_23, and Strep_24, are capable to inhibit the growth of fungal pathogens in dual culture under *in vitro* conditions. Among them, Strep_22 is the best one, followed by Strep_24 and Strep_23. The high efficacy of Strep_22 is confirmed also in *in vivo* conditions. The strain successfully protects the root of wheat and tomato from the infection of the same fungal pathogens tested with bergamot, pomegranate, and EP5. Moreover, Strep_22 promotes significantly the growth of the wheat and tomato plants. This plant-growth promoting effect is also related to *Streptomyces* spp. metabolites. Indeed, most *Streptomyces* spp. are reported as plant-growth promoting *Streptomyces* (PGPSs). This wide production of secondary metabolites is related to the complexity of their genome, which is highly different among the species. *Streptomyces* spp. genome is rich in cluster of genes that produces secondary metabolites, also known as secondary metabolites biosynthetic gene cluster (smBGC). After genomic analysis, Strep_22 resultes to be *Streptomyces syringium*. Although no information is available about this species, the strongly related species *Streptomyces necropsis* is known for being an antifungal agent. In fact, the metabolomic analysis of *S. syringium* reveals that it can produce secondary metabolites with antifungal, antibacterial, herbicide, larvicide, insecticide, and even nematocide effect, like phthalates, diketopiperazine and indole-oxazole alkaloids.

In conclusion, plant-derived products and microorganisms belonging to the genus *Streptomyces* are strongly suitable for the management of biotic adversities of the crops. To confirm these results, a further step is to evaluate the plant powders and the microorganisms in open field.