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Improvement of nitrogen and phosphorus use efficiency in wheat by application of Plant Growth Promoting Bacteria as bio-fertilizers

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Abstract

Due to the increase in human population growth, the depletion of natural resources and the environmental pollution, it is necessary to raise agricultural productivity without enhancing environmental footprint. A sustainable intensification may offer the opportunity of increasing input efficiency by reducing fertilizer applications and agricultural greenhouse gas (GHGs) emissions. The issue of sustainability of crop production is even more acute in semi-arid and arid regions, such as Mediterranean arable lands, where drought and related biophysical factors create a fragile and unstable environment. In Mediterranean Basin, wheat is one of the most widely grown crop; in particular, durum wheat represents the main grain crop in terms of surface area and provides food security to a large population share.

The use of Plant Growth Promoting Bacteria (PGPB) as inoculants in agricultural soils might be a suitable technology for sustainable farming systems in accordance with the reduction of environmental pollution and the need to use nitrogen (N) and phosphorus (P) resources more efficiently. Autochthonous microorganisms naturally possess some mechanisms of "adaptive evolution" to win and overcome the stressful environmental conditions and are able to enhance plant growth and protect plants from diseases and abiotic stresses.

Within this context, firstly, a review on PGPB application in cereals for a sustainable intensification was carried out focusing on the interaction PGPB-wheat (**see Chapter 2**). In particular, the main topic of this review was the potential of autochthonous PGPB isolated from soils to enhance nutrient use efficiency. As few data are available on the interaction plant-PGPB isolated from durum wheat rhizosphere, the aim of this PhD thesis was focused on the selection of PGPB from durum wheat rhizosphere and on the evaluation of their effect on N and P nutrition in wheat. To achieve this aim, the following goals were pursued:

i) the phenotypic and genotypic characterization of autochthonous non-pathogenic PGPB from durum wheat rhizosphere to select *in vitro* the most promising strains able to improve nutrient use efficiency;

ii) the evaluation of the effect of the PGPB selected as the best nitrifying bacteria on durum wheat N use efficiency;

iii) the evaluation of the effect on wheat seedlings growth of PGPB with P-solubilizing and P-mineralizing combined capability.

In order to achieve the first goal, four-hundred seventy-four bacteria (mesophiles, spore-formers, pseudomonads, actinobacteria) were isolated from rhizosphere of durum wheat. The isolates were preliminary characterized for phosphate solubilization, NH_4^+ production, nitrification and

siderophores production; then, some quantitative analyses were carried out (production of IAA-indole acetic acid, NO_2^-/NO_3^- and P-mineralization) and used as input to select some promising isolates through a new approach based on median and quartiles. As a result, sixteen strains were selected and identified by 16S sequencing. The promising bacteria were tested as inoculants in wheat seedlings for a preliminary validation in soil. Among them, three strains (25A- *Bacillus*, 6P- *Stenotrophomonas*, and 20P- *Stenotrophomonas*) showed the best performances in terms of plant biomass and height and were selected to evaluate their effect on wheat nutrient use efficiency (**see Chapter 3**).

A first experiment in a growth chamber (**see Chapter 4**) was carried out to evaluate the effect of PGPB application on durum wheat N use efficiency, by using the best nitrifying isolates (6P- *Stenotrophomonas* and 20P- *Stenotrophomonas*) which were also able to produce NH_4^+. To this aim, two genotypes (Saragolla and Simeto) were grown under controlled conditions to evaluate the capacity of the isolates to establish and survive in rhizosphere and their effect on N-uptake (UPE) and use efficiency (NUEP). In Saragolla both strains improved UPE and NUPE; on the contrary, in Simeto only 20P strain showed a positive effect.

A further pot experiment (**see Chapter 5**) was carried out to investigate the capacity of the best P- solubilizing and P- mineralizing isolates (12A- *Bacillus* and 25A- *Bacillus*) to promote the growth of wheat seedlings in low P soil. To this aim, durum and bread wheat plants were grown in low P soil to evaluate: i) the effect of bacterial isolates on root system architecture; ii) the expression of key genes involved in the P starvation response and Pi uptake, *IPS1* and *TaPT6* transporter. The IPS1 gene is highly responsive to P deficiency, and its transcript level is a good marker for P deficiency responses and has been shown to be involved in P homeostasis. PT6 is a high- affinity Pi transporter gene playing a dual role in Pi uptake from the soil and Pi translocation inside the plant. One isolate, 12A- *Bacillus*, significantly increased root length, surface area and biomass. These results might be explained by the capacity of 12A strain to produce indole-3-acetic acid (IAA) in addition to the P mineralizing and P solubilizing capability. Furthermore, an enhanced shoot dry weight and shoot P content was reported maybe because of combined capacity to increase root length and surface and to mineralize and solubilize P.

Further experiments under field conditions and under different environments will be necessary to validate the effect of the selected strains on wheat nutrient use efficiency.

1. Introduction

1.1 Improvement of natural resources efficiency

Despite the ever-growing world's population, the rate of growth in agricultural production is expected to fall to 1.5% between now and 2030 and further to 0.9% between 2030 and 2050, compared with 2.3% per year since 1961. In order to feed the growing population and to provide food security to the 870 million now chronically undernourished, food production must increase by 70%; in particular the annual demand for major grain crops, will need to rise to about 3 billion tonnes (Powell *et al*. 2012; Ray *et al*. 2013; FAO 2015). However, in 2018,FAO forecasts world cereal production at 2,6 million tonnes but at this level global production would still be down 40.6 million tonnes (minus 1.5 %) on a yearly basis (FAO 2017).

The increase of agricultural production can be achieved by: (i) increasing arable land (agricultural expansion); (ii) increasing yields (agricultural intensification). According to FAO (2009), the 90% (80% in developing countries) of the growth in crop production will come from by agricultural intensification, in particular higher yields and increased cropping intensity. The remaining 10% will come from by expanding arable land. At global scale, considerable land reserves could be converted to arable land however some of them have important ecological functions, which would be lost, or are located in just few countries in Latin America and sub-Saharan Africa where lack of access and infrastructure could limit their use in the short term (FAO 2015). In addition, the conversion of natural habitats to agriculture or deforestation may result in very significant releases of greenhouse gases (GHGs) into the atmosphere, in major biodiversity losses and can affect the ability of land to store water and prevent flooding (Godfray and Garnett 2014). Furthermore, an increasing number of countries are reaching alarming levels of water scarcity and are likely to worsen because of climate change in many regions (FAO 2015). Biodiversity losses, also have repercussions for global food security, reducing options for breeding new crops and plant varieties that may allow food systems to better adapt to climate change.Contrary to the aboveground diversity, the functional consequences of belowground biodiversity loss need to be more investigated since it represents one of the largest reservoirs of the total biological variety. Indeed soil biodiversity loss may significantly affects multiple ecosystem processes such as biogeochemical cycles and eco-evolutionary dynamics in plant and aboveground communities in response to current and future environmental global change (Bardgett *et al*. 2014).

Intensifying food production causes the well-known environmental problems because yields are raised through the application of fertilizers leading to the GHGs emission, even though, several studies suggest these are fewer than GHGs emission coming from converted land (Godfray and

Garnett 2014). FAO's data (http://www.fao.org/faostat/en/#data/GT) show that GHGs emissions due to the application of fertilizers represent the fastest growing emissions source among all agriculture sectors (enteric fermentation, manure management, rice cultivation, synthetic fertilizers, manure applied to soils, manure left on pastures, crop residues, cultivation of organic soils, burning of crop residues, burning of savanna, energy use). Additionally, an excessive use of these compounds causes leaching, pollution of water resources with severe consequences to the environment and human health.

The agricultural GHGs emissions are mainly associated with nitrogen (N) fertilizers and consist of direct and indirect N_2O emissions from N added to soil by farmers. Direct N_2O emission is produced by microbial processes of nitrification and denitrification taking place on the application site. The indirect N_2O emission is produced by: (i) a portion of volatilized ammonia (NH_3) deposited on soil and in water and subjected to nitrification process and (ii) a portion of nitrate (NO_3^-) that leaches and denitrifies (Snyder *et al*. 2009).

In addition to GHGs emissions, the agricultural intensification, could lead to the overexploitation and unsustainable use of natural resources, and the environment degradation leading to ever-growing competition for the remaining available resources, triggering soil nutrient depletion, erosion, desertification, depletion of freshwater reserves and aboveground and belowground biodiversity lossess (FAO 2015, FAO 2017).

Achieving a yield increase reducing negative impacts on the environment is not easy to obtain, but if little can be done to mitigate emissions from land conversion, a "sustainable intensification" approach of arable lands may offer the opportunity of increasing farm inputs (fertilizers, water, etc.) use efficiency and further reducing emissions (Godfray and Garnett 2014). Hence, a huge experimentation is requested to find out which production and farming techniques are the most effective and in which contexts. Current crop production systems vary widely. There are many farming systems with an eco-friendly and more sustainable approach than traditional production practices in terms of environmental impacts (Pisante *et al*. 2012).

The issue of crop production sustainability is more acute in semi-arid and arid regions, such as Mediterranean arable lands, where drought creates a fragile and unstable environment for crop production (Ryan *et al*. 2008). The increase in temperature (mainly due to the global warming and hence to GHGs emissions) and the change in precipitation predicted by common climate models for the Mediterranean Region, will affect water and the other resources availability, heavily influencing the future crop production. Rainfall patterns are less predictable than temperature, although it is likely that the frequency of heavy precipitation will increase in many regions (Stocker *et al*. 2013), leading

to run-off and therefore reducing water availability to crops while increasing the risk of soil erosion. At the same time, the frequency of drought stress is expected to increase in many regions (Hochman *et al*. 2009). Combined heat and drought stress is generally more detrimental than each stress alone (Pradhan *et al*. 2012) and is much harder to control in field trials.

1.2 Wheat: a staple crop to meet the world food demand

Wheat is currently the most widespread grain crop. It is grown on about 219 million hectares all over the world and with a worldwide production of 771 million tonnes (FAOSTAT 2018_ http://www.fao.org/faostat/en/#data). Wheat is the basic staple food for humans providing the 18% of their daily intake of calories and 20% of proteins. Roughly 90 to 95% percent of the wheat cultivated in the world, is common or bread wheat (*Triticumaestivum* L.) mainly used for manufacturing bread and biscuits; the rest is mostly durum wheat (*Triticumturgidum* L. subsp. *durum*) (Vita *et al*. 2016; Royo *et al*. 2017). Durum wheat isgrown on about 13 million hectares, about 60% of them located in the Mediterranean Basin, where it is considered a typical crop and mostly used for the production of pasta, couscous and bulgur (Royo *et al*. 2017). The first European producer of durum wheat is Italy (Vita *et al*. 2016) with a global production of 4.2 million tons and a surface of 1.27 million hectares. Most of durum wheat production comes from Southern Italy where Apulia Region has the largest cultivated surface in Italy (ISTAT 2018_ http://agri.istat.it).

Under climate change scenarios, wheat yields is destined to decline by 17% by the end of the century (IPCC 2014). In particular, data indicate that for each Celsius degree increase in global mean temperature, wheat grain yields may decline about 6% (Reynolds *et al*. 2016). Nevertheless, to meet the world food demand, global demand for wheat is projected to increase by 60% by 2050 (FAO 2017). However, according to the last FAO forecasts, the rate of wheat production are 0.4% below the 2017 estimation (FAO 2018).

The most important quality feature for wheat is grain protein content that is influenced by climatic parameters, *cultivar*, nitrogen fertilizer rate, time of nitrogen application, residual soil nitrogen and available moisture during grain filling (Rharrabti *et al*. 2003; Tamang *et al* 2017). To achieve the target concentration of proteins in grains, a high level of nitrogen fertilizer has been supplied in wheat production (Henry *et al*. 2016; Tamang *et al*. 2017).

In Mediterranean environments, grain maturation usually occurs under high temperature and terminal drought. In this environment, climate changes such as increased atmospheric CO_2 concentration and average temperature, limited water supply and higher frequency of heat waves impose a significant challenge to maintain wheat grain production and quality. Overall, under

elevated CO_2 wheat yield can increase but grain protein concentration decreases. The reduction in protein percentage due to elevated CO_2 combined with high temperature reducing glutenin/gliadin ratio is likely to have an overriding impact on dough functional properties and baking properties of bread (Nuttal *et al*. 2017). As a consequence, grainproduction and quality are likely to become increasingly variable and mitigation strategies are needed to reduce these variability.

Moreover, accounting for the negative effect of climate change and its future forecasts, particularly in semi-arid regions, significant cereal price rises are predicted to increase of about 60-97% by 2050 (Keating *et al*. 2010; Rosegrant *et al*. 2013).

1.3 Strategies for a sustainable crop production

A yield increase with lower negative impacts on the environment is difficult to achieve. A set of farming practices for sustainable production (such as no or minimum mechanical soil disturbance, maintenance of soil mulch cover, and diversified cropping) referred to as Conservation Agriculture (CA) has the potential to mitigate climate change by: (i) a more efficient use of fertilizers, agrochemicals and water; (ii) a better resilience to abiotic and biotic stresses (Pisante and Kassam 2017).

Alternatively, innovative agricultural techniques, known as Precision Agriculture, aim at improving production and reducing environmental pollution through a site-specific resources management related to field variability (Pisante *et al*. 2012). This agricultural system allows application of nutrients, water, and pest control measures to meet the specific requirements at each site within a field, increasing fertilizers use efficiency and reducing the nitrate leaching with site-specific management.

Also, biotechnologies have been proposed as smart strategies for the crop management by improving the nutrient uptake efficiency, controlling biotic adversity and reducing the use of fertilizers (Glick 2012; de Souza *et al*. 2015). Among them, the application of microbial inoculants, called bio-fertilizers, is a promising technology for sustainable farming systems to reduce the use of conventional inorganic fertilizers mainly N and P fertilizers. It is known, that both Arbuscular Mycorrhizal Fungi (AMF) and Plant Growth Promoting Bacteria (PGPB) alone or in combination can serve as bio-fertilizers as they are able to fix N, help to access nutrients such as P and N from organic fertilizers and soil stocks, improve drought tolerance and plant health or increase salt tolerance (Pellegrino *et al*. 2015; Saia *et al*. 2015; Dal Cortivo *et al*. 2017; Schütz *et al*. 2018).

1.4 Direct and indirect mechanisms of action of PGPB

In order to reduce the risk of the eutrophication of water resulting from nutrient run-off, the GHGs emissions, which is a major concern with N fertilizers (Snyder *et al*. 2009) and considering that phosphate rock, the source of P fertilizer, is a finite natural resource, there is a need to enhance N and P fertilizers use efficiency. On a worldwide scale, the demand for N and P fertilizers is increasing, reaching an estimated120 million tonnes of elemental N and 47 million tonnes of P_2O_4 by 2018 (Heuer *et al*. 2017). Despite the increase in N and P fertilizers demand,for all cereals, N and P use efficiency (NUE and PUE respectively) have been estimated to be as low as 33% and 16% (Hawkesford 2014; Dhillon *et al*. 2017) respectively. NUE was evaluated through several methods in the literature (reviewed in Good *et al*. 2004). According to an extensively used definition, plant NUE is defined as the grain yield produced per unit of applied N fertilizer. It is an integration of N uptake efficiency (NUpE) and N utilization efficiency (NUtE) defined respectively as the capacity of plants to acquire N from the soil and the fraction of plant-acquired N to be converted to total plant biomass or grain yield (Cormier *et al*. 2016; Ercoli *et al.* 2013).Some authors considered another measure of NUE: the NUEP, defined as grain protein per unit of available N (Le Gouis *et al*. 2000; Giuliani *et al*. 2011a). Also NUEP can be partitioned in the UPE and N utilization efficiency (NHI, grain N per unit of N in plant). Likewise, PUE can be divided into P acquisition efficiency (PAE) and P internal utilization efficiency (PUTIL). PAE refers to the ability of plants to take up P from soils, whereas PUTIL is the ability to produce biomass or yield using the acquired P (Mathur 2014).

It is known that use of PGPB as inoculants in agricultural soils might be a technology for sustainable farming systems according with the reduction of environmental pollution and the need to use more efficiently N and P resources because of the capacity to enhance plant growth and protect plants from disease and abiotic stresses. *Agrobacterium*, *Allorhizobium*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Enterobacter*, *Erwinia*, *Exiguobacterium*, *Flavobacterium*, *Mesorhizobium*, *Micrococcus*, *Providencia*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Xanthomonas* are the main microorganisms with plant growth promoting properties (Yadav *et al*. 2017; Karnwal 2017). Interactions between plants and bacteria may occur both in phyllosphere (aerial plant surfaces) or rhizosphere (area surrounding the roots), but the environmental conditions for both sections are different. Phyllosphere is exposed to frequent changes of temperature, humidity, intensity light, etc., with consequent changes in nutrients availability. Rhizosphere provides a better protection from changes in temperature and light intensity and guarantees a higher abundance of nutrients. PGPB can interact with host plant as free-living bacteria, symbiotic (that form a symbiotic relationship with roots), endophytes (that

colonize only a portion of interior tissue of plant having a direct access to organic compounds), and cyanobacteria (formerly called blue-green algae) (de Souza *et al*. 2015).Optimizing plant-microbial partnerships is a critical task due to the complexity of plant-microorganism and microorganism-microorganism interactions, and the dependency of those interactions on environmental conditions. Thus, a better understanding of complex interactions among plant genotypes, environmental conditions and microbiome structure provides indispensable information in breeding programs (Toju *et al*. 2018).

PGPB can act by direct and indirect mechanisms and typically, more than one mechanism is used for promoting plant growth. Direct mechanisms comprise: biological nitrogen fixation (BNF), production of indolic compounds (such as indolic-3 acetic acid, IAA) and siderophores, synthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase that modulate the level of plant hormones, P-solubilization and P-mineralization. In addition, through indirect mechanisms like the production of siderophores which inhibit the growth of plant pathogens, antibiotic and antifungal metabolites, or the induction of resistance, PGPB prevent harmful effects of plant pathogens (Glick 2012; de Souza *et al*. 2015; Di Benedetto *et al*. 2017).

A critical issue of the use of PGPB as inoculants is the timing of inoculum application. In plant microbiome assembly, differences in the colonization processes, such as the order of species' arrival, can result in large differences in community structure. Indeed, "early colonizers" can use space and resources earlier than other microorganisms and/or can produce physical barriers and/or antibiotics that slow colonization of subsequent microorganisms on the plants exclude competitors from endospheres, phyllospheres or rhizosphere(Toju *et al*. 2018).

Table 1 offers an overview of the main direct and indirect mechanisms by which PGPB act.

Table 1: PGPB direct and indirect mechanism of action (modified from Di Benedetto *et al*. 2017)

DIRECT MECHANISMS	**MICROORGANISMS**
PHOSPHATE SOLUBILIZATION Some rhizosphere colonizing bacteria enhance P availability by making soluble, insoluble phosphate inorganic compounds.	Bacteria: *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Serratia* together with *Pantoea* and *Klebsiella. Rhodococcus*, *Arthrobacter*, *Chryseobacterium*, *Gordonia*, *Phyllobacterium* and*Delftia*. Rhizobia: *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*
ORGANIC PHOSPHORUS MINERALIZATION The microbial organic P mineralization is carried out by phytase able to release P from	

organic materials in soil stored as phytate, or by phosphatase.	
NITROGEN FIXATION Atmospheric nitrogen (N_2) is not accessible to most living organisms but through Biological Nitrogen Fixation (BNF) it is reduced to ammonia (NH_3). BNF is performed by symbiotic or non-symbiotic N fixing microorganisms.	Non-symbiotic: *Cyanobacteria*, *Acetobacter, Achromobacter, Alcaligenes*, *Anabaena*, *Arthrobacter*, *Azoarcus*, *Azomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Corynebacterium*, *Derxia*, *Enterobacter*, *Gluconoacetobacter diazotrophicus,Herbaspirillium* sp. *Klebsiella*, *Nostoc,Pseudomonas*, *Rhodospirillum*, *RhodoPseudomonas* and *Xanthobacter*. Symbiotic: *Rhizobia*, *Frankia*.
INDOLEACETIC ACID (IAA) IAA performs many functions: (i) affects cell division, extension and differentiation of plants, (ii) stimulates the germination of seeds and tubers, (iii) exerts a control on processes of vegetative growth, (iv) concurs to increase the rate of xylem and root growth, (v) initiates formations (lateral and adventitious) of the roots, (vi) modulates responses to light, gravity and fluorescence, (vii) affects photosynthesis, pigment formation, biosynthesis of various metabolites, (viii) resists to stressful conditions.	Several microbial species are able to produce IAA, through five biosyntetic pathways: (1) Saprophytic species of *Agrobacterium*, *Azospirillum*, some species of *Bradyrhizobium*, *Enterobacter*, *Erwinia herbicola*, *Klebsiella, Pseudomonas* and *Rhizobium* through via indole-3-pyruvic acid and indole-3-acetic aldehyde; *(2) Pseudomonads and Azospirilla* through the conversion of tryptophan into indole-3-acetic aldehyde with tryptamine as intermediate compound; (3) *Agrobacterium tumefaciens*, *E. herbicola*, *P. fluorescens,Pseudomonas syringae* and *Pseudomonas putida* through the formation of indole-3-acetamide; (4) *Cyanobacterium* (*Synechocystis sp.*) through the formation of indole-3-acetonitrile; (5) *Azospirilla* and *Cyanobacteria* are also able to produce IAA through a tryptophan-independent pathway.
SIDEROPHORE PRODUCTION Bacteria can overcome the nutritional Fe limitation by using chelator agents called siderophores. Siderophores are defined as low-molecular-mass molecules (< 1000 Da) with high specificity and affinity for chelating or binding Fe^{3+}, followed by the transportation and deposition of Fe within bacterial cells. Various studies have shown that siderophores are largely produced by bacterial strains associated with plants. Some plants can bind and release Fe from bacterial Fe^{3+}-siderophore complexes, and use the iron for growth.	*Bradyrhizobiumjaponicum*, *Rhizobium leguminosarum* and *Sinorhizobium meliloti* are the main microorganisms involved in siderophore production. Among Gram-negative bacteria, siderophore-producing belong to the *Pseudomonas* and *Enterobacter* genera, while *Bacillus* and *Rhodococcus* are the most representative among Gram-positive bacteria.

ACC DEAMINASE ACTIVITY 1-aminocyclopropane-1-carboxylate (ACC) deaminase is a bacterial enzyme involved in reducing the level of ethylene in plants. PGPB in response to tryptophan or other small molecules (see IAA section) synthetize and secrete IAA. This bacterial IAA, together with endogenous plant IAA, stimulate plant growth or induce the synthesis of the plant enzyme ACC synthase which converts the compound *S*-adenosyl methionine (SAM) to ACC, the immediate precursor of ethylene.	*Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* etc.
INDIRECT MECHANISMS	**MICROORGANISMS**
SIDEROPHORE PRODUCTION By sequestering iron, PGPBs siderophore-producing reduce the availability of this element necessary for the growth of pathogens. Siderophore mediated iron scavenging in Gram-negative transport is better studied PGPB than Gram-positive PGPB.	*Bradyrhizobiumjaponicum*, *Rhizobiumleguminosarum* and *Sinorhizobium* meliloti are the main microorganisms involved in siderophore production. Among Gram-negative bacteria, siderophore-producing belong to the *Pseudomonas* and *Enterobacter* genera, while *Bacillus* and *Rhodococcus* are the most representative among Gram-positive bacteria.
PRODUCTION OF ANTIBIOTIC AND ANTIFUNGAL METABOLITES Phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide production.	*FluorescentPseudomonads*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Paenibacillus*, and *Streptomyces*.
PRODUCTION OF LYTIC ENZYMES Chitinases, cellulases, β-1,3 glucanases, proteases, and lipases lyse a portion of the cell walls of many pathogenic fungi.	*Botrytis cinerea*, *Sclerotium rolfsii*, *F. oxysporum, Phytophthora* spp., *Rhizoctonia solani,* and *Pythium ultimum*.
COMPETITION FOR NUTRIENTS	*Actinobacteria, Az. brasilense, Bacillus amyloliquefaciens Bacillus pumilus Bacillus sp., Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Brevibacillus, Enterobacter sp., Jeotgalibacillus, Lysinibacillus, Paenibacillus, Paenibacillus polymyxa, Pseudomonas sp., Pseudomonas chlororaphis, P. fluorescens, Pseudomonas aeruginosa, Terribacillus*

INDUCED SYSTEMIC RESISTANCE (ISR) "Induced resistance" is referred to the induced state of resistance in plants triggered by inducers (biological or chemical); this state protects non-exposed parts against possible attack by pathogenic microbes and herbivorous insects.	*Pseudomonas fluorescens*, *Serratia marcescens*, *Pseudomonas protegens*.

1.5 PGPB as bio-fertilizers to improve nutrient use efficiency

When PGPB enhance nutrient availability to the plant through either N fixation, or mineral solubilisation (phosphorus, potassium), sequestering iron, inducing increase in root surface area, they may collectively be termed as bio-fertilisers. According to the definition of Vessey (2003), "biofertilizer is a substance containing living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant".

PGPB as commercial bio-fertilizers

There is growing interest on the use of PGPB in cereals and various studies demonstrate their beneficial role in the growth and yields of crop species like maize (*Zea mays*), rice (*Oryza sativa*) and wheat (*Triticum aestivum* L.) which represent the three first crops in terms of production in the world (FAO 2015). *Pseudomonas*, *Burkholderia*, *Azospirillum* and *Herbaspirillum* are the most frequently PGPB used as bio-fertilizers for these crops. In most cases, their use as commercial formulations statistically increases crop parameters like grain production, root and shoot length or plant weight in pot or field experiments (for a review see Pérez-Montaño *et al*. 2014). For instance, a strong increase in total plant and grain weight was reported when maize plants were inoculated with *Burkholderiacepacia*, *Azospirillumbrasiliense* and *Herbaspirillum seropedicae* acting as free-living nitrogen-fixing PGPB in pot and field experiments comparing with plants grown without N (Riggs *et al*. 2001). Rosas *et al*. (2009) studied the effect of *Pseudomonasaurantiaca* SR1- formulation on maize in field treatments that included P and N fertilization. When inoculated with the SR1 strain, plants showed a significant increase in growth parameters and higher yields with lower fertilization doses than with conventional strategies. Likewise, in Krey *et al*. (2013) field application of *Pseudomonasfluorescens* DR54 improves maize growth and soil P pools in P-deficient growth condition suggesting the use of this commercial formulation on P poor soils.

Similar effects were reported in rice. In de Salamone *et al*. (2012) the inoculation of paddy rice (*cv* Supremo 13) with commercial formulation containing *A. brasilense* and *P. fluorescens* increased aerial biomass production, harvest index, and grain yield in field experiments. Also, enhanced emergence and seedling vigor of rice was reported under *Bradyrhizobium* strains inoculation (Baset and Shamsuddin 2009).

Higher grain yields, harvest index and protein content with lower fertilizer doses, were observed, in both pot and field experiments, when wheat seeds were inoculated with *P. fluorescens* SR1 strain (Rosas *et al*. 2009). More recently, Dal Cortivo *et al*. (2017) investigated whether a commercial bio-fertiliser containing a consortium of PGPR and N-fixing bacteria (*Azospirillum* spp., *Azoarcus* spp. and *Azorhizobium* spp.) affects shoot and root growth, N accumulation and grain yield in common wheat (*Triticumaestivum* L.). The main result of this study was the finding that the crop exhibited greater N accumulation, so some beneficial environmental effects in terms of reduced N losses from agricultural ecosystems can be expected from the application of PGPRs.

Furthermore as reported in Kumar et al. (2014), a combination of a common fertilizer (di-ammonium phosphate) and commercial PGPB (*Azotobacterchroococcum* and *Bacillussubtilis*) increased nutrients availability and productivity of wheat more than the fertilizers applied alone. The same fertilizers when entrapped in an organic matrix further enhanced nutrients availability in soil as well as uptake in plants. Also, the data indicate that even the lower dose of conventional chemical fertilizers in combination with microbial bio-fertilizers and organic matrix can be more effective for the enhanced productivity of wheat.

PGPB as autochthonous bio-fertilizers

In literature, several reports are available on the application of commercial PGPBon growth and yield of different cereal crops; on the contrary, less effort have been made on the study of the effect of autochthonous PGPB isolated from soil. A critical issue of the use of allochthonous formulates is that they could not possess some mechanisms of adaptive capacity to win the stressful environmental conditions in different ecological environments (Corbo *et al*. (2017)while autochthonous (native) strains naturally possess some mechanisms of adaptive evolution. The use of native strains as inoculants, isolated, characterized and selected for growth promoting traits of interest could represent an important tool to search for region-specific microbial strainsto achieve the maximum benefits in terms of fertilizer saving and plant growth promotion(Majeed *et al*. 2015) Thus, deeper investigation on the isolation and characterization of autochthonous strains is needed.In this context, the *in vitro* study of growth-promoting traits represents a critical phase to select microorganisms able to survive and establish in crop rhizosphere to be used as bio-fertilizers.

For example, in Montañez *et al*. (2012) a set of putative endophytic bacteria including *Pseudomonas*, *Herbaspirillum*, *Enterobacter* and *Burkholderia* were isolated from maize plants and characterized *in vitro* for IAA-production, siderophores production and P solubilization capability. Maize inoculation experiments showed significant increases in shoot dry weight under controlled conditions. Likewise, Zhao *et al*. (2014) isolated twelve bacterial strains showing different degrees of P solubilizing activity from maize rhizosphere. One isolate identified as *B.cepacia*, in addition toP-solubilizing activity was also able to promote the growth of maize plants when infected by pathogenic fungi. These results indicated that the isolate was a good candidate to be applied as a bio-fertilizer and a biocontrol agent.

In Manzoor *et al*. (2017) the capacity of PGPB isolated from maize rhizosphere to solubilize mineral phosphate and to produce IAA was reported. Also, five potential isolates were tested as inoculants in maize plants since their ability to increase P release capacity (mineralization) of insoluble RP in a greenhouse experiment. The overall effect of P solubilizing isolates with RP over RP alone on maize growth showed a relative increase in shoot and root fresh and dry weight, length and chlorophyll content. This study indicated that use of P solubilizing bacteria (PSB), and organic amendments with insoluble RP could be a promising management strategy to enhance P availability in soil pool and improve plant growth in intensive cropping systems.

In rice, some reports describe the isolation and characterization of free-living diazotrophic bacteria able to solubilize inorganic phosphate or to produce IAA in addition to fix atmospheric N. For instance, the application of these PGPB in pots and field experiments showed a statistically significant increase in grain production, root and leaf length or plant weight (Islam *et al*. 2009; Araújo *et al*. 2013). The identification and isolation of PGPB from rice, belonging to different groups and characterized for different plant growth traits were reported by Park *et al*. (2007), Beneduzi *et al*. (2008), Nathan *et al*. (2011), Dewangan *et al*. (2014) and Anitha *et al*. (2014).

As mentioned on the other cereal crops, the isolation and characterization of autochthonous PGPB was reported in wheat. Inoculation of wheat seeds with ACC deaminase producer *P. fluorescens* strain allowed the decrease of N, P and K fertilizer rates. These results indicated that these Pseudomonads could be used in combination with appropriate doses of fertilizers for better plant growth and reducing the use of fertilizers (Shaharoona *et al*. 2008). Majeed *et al*. (2015) studied and isolated plant growth-promoting bacteria from wheat rhizosphere. The authors demonstrated the positive effects of efficient N-fixing, P-solubilizing, and IAA-producing bacteria (*Bacillus* sp., *Stenotrophomonas* spp.) for enhancing growth and nutrient content of wheat under field conditions. The authors reported that these strains provided a significant increase in shoot and root length, and

biomass. Furthermore, a significant increase in shoot and root N contents was observed over the un-inoculated control. In Rana *et al*. (2012) ten wheat rhizosphere isolates for plant growth promoting traits (IAA, siderophore and NH_4^+ production, ACC deaminase activity and N-fixing ability) were screened. Among them three promising strains, identified as *Bacillus* sp., *Providencia* sp. and *Brevundimonas* sp. were found to enhance plant growth and yield, as well as enriching nutrients in plants. A positive correlation of yield (grain weight and panicle weight) and plant biomass as well as nutrient content (N, P) was recorded.

As emerged from the literature review reported above, to date, there are some papers dealing with the benefits of autochthonous PGPB in maize, rice and common wheat but at our best knowledge, few reports are available on the isolation and characterization of PGPB from durum wheat.

1.5.1 PGPB and N nutrition in wheat

The main way to maintain or restore N in soil is the application of mineral fertilizers. Because of the simplicity of its storage and handling, N fertilizers can easily be applied when plants need it most. Moreover, in production systems, over 50% and up to 75% of the N applied to the field is not used by the plant and is lost by leaching into the soil (Hirel *et al*. 2011). The N used in commercial fertilizers, as nitrate (NO_3^-) and urea $\{CO(NH_2)_2\}$, is very soluble and can run off into the surface water or flow into the groundwater with detrimental impact such as the eutrophication of freshwater. Nitrate loss from the soil can also lead to soil eutrophication: an excessive amount of nutrient can cause oxygen depletion in surface soil thus limiting the function of soil microorganisms including those involved in N cycle (Hirel *et al*. 2011).

Soil inoculation with PGPB can represent a smart tool to improve plant N nutrition in order to reduce the environmental pollution and N_2O emissions from N added to soil by farmers. These microorganisms may improve plant N uptake and yield by increasing root surface area and/or N availability in the rhizosphere by N fixing bacteria or nitrifying bacteria.

IAA-producing bacteria

Certain soil microorganisms may help the plants to take up N, even from nutrient deficiency soil, since their ability to synthesize and export organic compounds such as auxin. Indeed, it is reported that auxin level is usually higher in the rhizosphere, where high percentage of rhizosphere bacteria is likely to synthesize auxin as secondary metabolites because of the rich supplies of root exudates. IAA is the most abundant endogenous auxin produced and it has been recognized as an important factor contributing to the stimulation of root development in plants (Spaepen *et al*. 2007). In particular, branching through lateral and adventitious root formation represents an important element of the adaptability of the root system to N deficiency. Both are regulated by N availability

and hormonal signals (Bellini *et al*. 2014; Giehl and von Wirén, 2014), which act locally to induce root branching for increasing the surface area of the plant root system in the deeper layers for NO_3^- uptake, especially for post anthesis N uptake (Foulkes *et al*. 2009; Atkinson *et al*. 2014; Cormier *et al*. 2016). It is known, that the inoculation of roots of wheat plants with optimal concentration of *Azospirillum* enhances root proliferation mainly through an increase in lateral root and root hair formation confirming the role of IAA produced by *Azospirillum* in altering root architecture (Dobbelaere *et al*. 1999; Spaepen *et al*. 2008; Veresoglou and Menexes 2010; Spaepen *et al*. 2011).

Diazotrophic bacteria

The community of N fixers plays a key role for plant N nutrition (Hsu and Buckley 2009). Bacteria that fix atmospheric N_2 into ammonium (NH_3) are referred to as diazotrophic. Among the plant root-associated diazotrophs, the symbiotic bacteria of the Rhizobium legume system is the major contributor of biologically fixed N as compared to non-symbiotic N-fixing bacteria, NFB (Venieraki *et al*. 2011). Moreover, it is known, that free-living root-associated diazotrophic (non-symbiotic) bacteria are commonly present in the rhizosphere of wheat plants (Venieraki *et al*. 2011). In wheat and in other cereals, conversion of N_2 into NH_3 does not entail root-nodulating rhizobia, but it can be performed by other non-nodulating N-fixing bacteria and part of the N fixed may be acquired by the plant enhancing wheat yield as reviewed in Cormier *et al.* (2016). For example, Iniguez *et al*. (2004) reported that wheat plants inoculated with NFB *Klebsiellapneumoniae* 342 assimilated up to 49% of the plant N from the atmosphere through biological N fixation while plants grown under N deficient conditions inoculated with mutant strain, unable to fix N, showed signs of N deficiency.

Ammonia- producing and nitrifying bacteria

N acquisition by roots is strictly dependent on the availability of the N source itself, but about 90% of total N is present as soil organic matter. Therefore, the mineralization, i.e., ammonification and then nitrification, carried out by bacteria is crucial for plant N nutrition (Pii *et al*. 2015).

In accordance with information available in literature, both the ammonification and the nitrification capacity represent two important skills influencing N-Uptake Efficiency and in the selection of strains to be used as bio-fertilizers (Richardson *et al*. 2009; Cormier *et al*. 2016).

1.5.2. PGPB and P nutrition in wheat

It is known that the 50% of global cultivated land suffers from P deficiency because of insufficient P replacement into agricultural systems; furthermore, much of the soluble inorganic P that is used as chemical fertilizer is immobilized soon after it is applied becoming unavailable to plants (Heuer *et al*. 2017). Plants have evolved a set of adaptive mechanisms to respond to P

deficiency as: (i) the root exudation of organic acids (citrate, malate, and oxalate) to solubilize P complexes; (ii) the root exudation of phosphatases and phytases to access organic P; (iii) the development of high- and low-affinity Pi transporters for Pi uptake from the soil and internal distribution, as well as for storage and remobilization of Pi from the vacuole (Heuer *et al*. 2017).

Furthermore, different regulatory pathways involved in P deficiency response in plants have been described too. For example, a Pi-mediated signalling cascade, involving the Induced by Pi Starvation 1 (IPS1) gene, the small RNA miR399 and the genes *UBC24* and *PHO2*, is induced by P deficient soil conditions. IPS1 transcripts inhibit the action of miRNA399 silencing complexes on PHO2 mRNA (a ubiquitin E2 conjugase) thereby adjusting the transcript levels of PHO2, which is a key player in balancing Pi with respect to its supply and demand (Franco-Zorrilla *et al*. 2007; Briat *et al*. 2015; Kumar *et al*. 2017).

Concerning the environmental pollution, the P deficiency of soil, the limited availability and the non-renewable nature of rock phosphate, efficient use of P fertilizers has become an important issue of agriculture. The inoculation of soil with microorganisms able to mobilize the poorly available P could represent a promising strategy for the improvement of PUE. It is known that in soil and rhizosphere numerous microorganisms are able to release P from total soil P through solubilization or mineralization. Among them bacteria are more effective in P solubilization than fungi. P solubilizing bacteria (PSB) constitute 1 to 50%, while P solubilizing fungi (PSF) are only 0.1 to 0.5% in P solubilization potential (Mohammadi *et al*. 2012; Sharma *et al*. 2013). As reported for the interaction PGPB-N nutrition, these microorganisms may improve plant P uptake and yield by increasing root surface area.

P-solubilizing and P- mineralizing bacteria

The main mechanism of microbial P solubilization is the production of mineral dissolving compounds such as organic acids, siderophores, protons, hydroxyl ions and CO_2 (Rodríguez and Fraga 1999; Sharma *etal*. 2013). Organic acids together with their carboxyl and hydroxyl ions chelate cations or reduce the pH to release P (Alori et *al*. 2017). The excretion of these organic acids is accompanied by a reduction in pH leading to the acidification of the microbial cells and the surroundings, hence, P ions are released by substitution of H^+ for Ca^{2+} (Alori *et al*. 2017).

An alternative mechanism to organic acid production for solubilization of mineral P is the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase (Rodríguez and Fraga 1999; Sharma *et al*. 2013). Furthermore, it was reported that the assimilation of NH_4^+ within microbial cells is accompanied by the release of protons and this results in the solubilization of P without the production of any organic acids.

Concerning the mineralization of organic P, which represent the 50-70% of total P in soillargely stored as phytate, which is not available for plant use, it is carried out by phytase enzymes produced by P mineralizing bacteria (PMB). Other enzymes produced by PMB in the process of organic P mineralization are phosphatase enzymes. In fact, the major source of phosphatase activity in soil is considered to be of microbial origin as confirmed by the presence of a significant amount of microbial phosphatase activity detected in different types of soils (Rodríguez and Fraga, 1999). To date, very few reports documented phytate mineralization by PGPB while phytase producing rhizobacteria are well documented (Hussin *et al*. 2007; Shedova *et al*. 2008; Sharma *et al*. 2016).

PSB and PMB including various strains of *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Kushneria*, *Paenibacillus,Rhizobium*, *Rhodococcus*, *Serratia*, *Salmonella*, *Sinomonas*. P-solubilization and mineralization could coexist in the same bacterial strain (Tao *et al*. 2008).

Additionally, the P-solubilizing ability of actinomycetes has attracted interest in recent years because this group of soil organisms is capable of surviving in extreme environments (e.g. drought, fire.) and possesses other potential benefits (e.g. production of antibiotics and phytohormone-like compounds) that together with P-solubilizing ability could simultaneously benefit plant growth (Sharma *et al*. 2013; Alori *et al*. 2017).

Sarker *et al*. (2014) isolated, screened and characterized PSB from wheat and evaluated their efficacy in P nutrition in wheat. Upon screening, nine isolates showed varying levels of P-solubilizing activity. In *vitro* wheat seedling bioassay with two PSB isolates and varying sources of P, revealed that both isolates significantly enhanced seedling growth (shoot and root length, shoot and root dry weight) and nutrient contents (%N, %P) in plant tissue compared to no- PSB control. The results suggested that the isolates might be useful for improving P nutrition in wheat in soils with low available P.

Ogut *et al*. (2016) reported a two years field experiment conducted using wheat (*Triticumaestivum* ssp. vulgare L.) as the test plant for the evaluation of P-solubilizing microorganisms.Inoculations with *Bacillus* sp. #189 significantly increased plant P content and biomass. Increases in plant potassium (K), magnesium (Mg), zinc (Zn), and manganese (Mn) contents were also observed. These results suggested that promising P-solubilizing microorganisms might also show the potential to improve micronutrient uptake.

Sial *et al*. (2018) conducted a study to compare the effect of different P fertilizer rates and bacterial inoculants (*Bacilluspolymyxa*) on wheat growth and yield. Among the tested P fertilizer rates, the highest plant height, number of spikelets per spike, number of grains per spike, grain yield plant^{-1} and 100-grain weight were recorded in plots fertilized with 75 kg P_2O_5 ha^{-1}. More interestingly,

the yield performance observed in PSB inoculated pots was the same observed under application of 25 kg P_2O_5 ha^{-1}. Hence, it can be concluded that PSB inoculation can contribute to wheat yield potential to some extent in limiting farming system.

IAA-producing bacteria

As reported for N uptake, the modification of root architecture by IAA-producing bacteria is widely recognized as an important strategy for better P uptake under P deficient conditions, increasing the surface area of the plant root system in the surface layers. Many papers report the multiple benefits of PGPB inoculation in enhancing P as well as the production of IAA in wheat (Dobbelaere *et al*. 1999; Spaepen *et al*. 2008; Veresoglou and Menexes 2010; Spaepen *et al*. 2011, Rana *et al.* 2012; Majeed *et al*. 2015).

1.6 Aims of the research

In literature, it is reported that the use of PGPB as bio-fertilizers has gained importance in order to reduce the environmental pollution, the agricultural GHGs emission and to preserve the non-renewable nature of rock phosphate.

The aim of this research was to screen autochthonous PGPB able to survive and establish in wheat rhizosphere and to evaluate their capacity to improve nitrogen and phosphorus use efficiency in wheat.

To achieve this aim, the following goals were pursued:

i) thephenotypic and genotypic characterization of autochthonous non-pathogenic PGPB from durum wheat rhizosphere to select *in vitro* the most promising strains able to improve nutrient use efficiency;

ii) the evaluation of the effect on durum wheat N use efficiency of the application of PGPB selected as the best nitrifying bacteria;

iii) theevaluation in wheat, of the effect on plant growth of the application of PGPB with P-solubilizing and P-mineralizing combined capability.

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Chapter 2

The role of Plant Growth Promoting Bacteria in improving nitrogen use efficiency for sustainable crop production: a focus on wheat

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Abstract

Due to the increase in both human population growth and environmental pressure, it is necessary to raise agricultural productivity without enhancing environmental footprint. Within this context, soil inoculation with PGPB (Plant Growth Promoting Bacteria) may be considered a promising tool of integrated management systems. In particular, PGPB may improve plant growth either directly, by facilitating resource use or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogenic agents. PGPB comprise different functional and taxonomic groups of bacteria like *Pseudomonas, Bacillus, Rhizobium* and others. Their ability to either mobilize mineral or organic bound nutrients from the pedosphere or to fix atmospheric N_2 and make it available to the plants, is a crucial feature in their application. In literature some data are available on the use of commercial PGPB, while less efforts have been made on the study of the effect of autochthonous PGPB isolated from soils on sustainability of cropping systems; thus a literature survey on these aspects was carried out with special focus on wheat, a staple food for a large part of world population. In particular, the main topic of this review is the potential of PGPB to enhance use efficiency of agro-environmental resources focusing on the interaction PGPB-wheat for improving nitrogen useefficiency.

Keywords: PGPB; plant-bacteria interaction; rhizosphere; N-uptake; wheat; NUE

1. Introduction

The Food and Agriculture Organization of the United Nations (FAO) reported a projection of world population of 9.1 billion in 2050. The demands for major grain crops, such as wheat, are projected to 70% increase by 2050 [1] mainly through the increasing in crop intensity. In the past decades, agricultural practices aimed at maximizing yields mainly by increasing fertilization, without considering the socio-economic and ecological consequences [2,3]. Indeed it would be of interest to ensure food production using sustainable technologies that reduce environmental impacts including ecosystem degradation and high greenhouse gas emissions. Sustainable intensification was defined as "maximization of primary production per unit area without compromising the ability of the system to sustain its productive capacity" [4]. The issue of primary production sustainability is more acute for wheat, which is the main cereal crop used for human consumption in many areas worldwide, it provides 50 percent of humanity's dietary energy supply with corn and rice [5]. Also durum wheat (*Triticum turgidum* L. subsp *durum*), a crop well adapted to Mediterranean basin, is a staple food for a part of world population being mainly used for pasta production [6].

Among fertilizers, nitrogen is the nutrient that is most susceptible to loss and its availability is affected by soil type, tillage, N-source, crop rotation and precipitation [7]. Moreover, its recovery by the crop is usually less than 50% of the applied amount [8]. However, present concerns about crop and environmental sustainability are putting added emphasis on increasing the nitrogen use efficiency (NUE) of crops. Improving NUE is among the main targets of crop research for Mediterranean environments [8].

Researchers, farmers, agricultural policy are focusing their attention towards potential innovative biotechnological solutions with lower environmental impact. Green biotechnologies have been proposed as new strategies for the management of the crops by improving the nutrient uptake efficiency, controlling biotic adversity, reducing the use of fertilizers, etc [9,10]. It is known that some microorganisms (called Plant Growth Promoting Bacteria, PGPB) are able to influence biological nitrogen fixation, solubilize phosphate, produce phyto-hormones and other molecules, favor positive mycorrhizal-plant interactions and defend the plants from pathogenicbacteria.

In particular, soil inoculation with PGPB is a promising tool of integrated management systems to increase the efficiency of plants' use of nutrients (from either soil or fertilizers) through microbial technology and the sustainability of the cropping systems. PGPB are around/on the root surface and comprise different genera, like *Azospirillum, Azotobacter, Nitrobacter*, largely

studied but few proficient to colonize root, and other genera such as *Bacillus, Pseudomonas, Bradyrhizobium, Acinetobacter, Klebsiella, Mesorhizobium, Rhizobium,* etc. that are proficient to colonize the root surface, survive and compete with other microbiota [11].

After an initial overview on environmental problems due to the crop intensification, this study reports (i) the use of PGPB to improve the sustainability of cropping systems; (ii) how PGPB interact with plants (in particular with wheat) for the improvement of resources uptake efficiency; (iii) a focus on NUE inwheat.

2. Greenhouse Gas (GHG) Emissions from AgriculturalSystem

The annual demand for major grain crops, such as wheat, will need to rise to about 3 billion tons from 2.1 billion today [4,12]. In opposition with the rapid increase of the world population, the rate of growth in agricultural production is expected to decrease as a consequence of climate changes. In particular, FAO's data show that annual crop production is expected to fall to 1.5% between now and 2030 and further to 0.9% between 2030 and 2050[4].

Climate models predict a mean increase in temperature from 1.0 to 3.7 °C with an increase in frequency of heat waves by the end of 21st century. Similarly, for rainfall patterns longer drought periods are predicted alternating with heavy rainfall, which will lead to flash floods events. In Europe, climate change is considered the main reason for decreasing yield growth rate in wheat. In particular, summer precipitations are predicted to decrease and heat waves will become more common and severe, with a negative impact on crop productivity [12,13].

According to FAO the 90% of the growth in crop production will come from the agricultural intensification in particular from higher yields and increased cropping intensity. The remaining 10% will come from expanding arable land.

The agricultural intensification needed to increase crop production and food security, is linked to an increase in greenhouse gas (GHG) emissions. GHGs absorb infrared radiation in the atmosphere, trapping heat and warming the surface of the Earth. FAO's data show that GHGs emissions from agriculture including all the emissions produced in the different agricultural sectors (enteric fermentation, manure management, rice cultivation, synthetic fertilizers, manure applied to soils, manure left on pastures, crop residues, cultivation of organic soils, burning of crop residues, burning of savanna, energy use) have been estimated at 10% of total global emissions [14]. The main GHGs associated with agriculture are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) and the largest source of GHGs emissions within agriculture is enteric fermentation in ruminants, which is a major source of CH_4 produced and released by

livestock during ruminant digestion. In terms of the magnitude of emissions, it accounts for 40% of the whole agricultural GHGs emissions and is followed by the manure left on pasture (16%), the use of synthetic fertilizers (13%) and the rice cultivation (10%)[14].

However while emissions from ruminant fermentation have increased by 8% between 2004 and 2014, emissions generated during the application of synthetic fertilizers, have increased by 20% since 2004 representing the fastest growing emissions source in agriculture (Figure1).

In particular, world total annual emissions from synthetic fertilizers have increased from 548 $MtCO_2$ in 2004 to 659 $MtCO_2$ in 2014 and it was estimated that, in 2014, about 108 million tons of nitrogen fertilizers have been used worldwide and the 50% of it was used for cereal crops [15].

Emissions from synthetic fertilizers consist of direct and indirect N_2O emissions from nitrogen added to agricultural soils by farmers. Direct N_2O emission is produced by microbial processes of nitrification and denitrification taking place on the addition site. The indirect N_2O emission is produced by: (i) a portion of volatilized ammonia (NH_3) that will be deposited on soil and in water and be subjected to nitrification process and (ii) a portion of nitrate NO_3^- that leaches and will be denitrified [16].

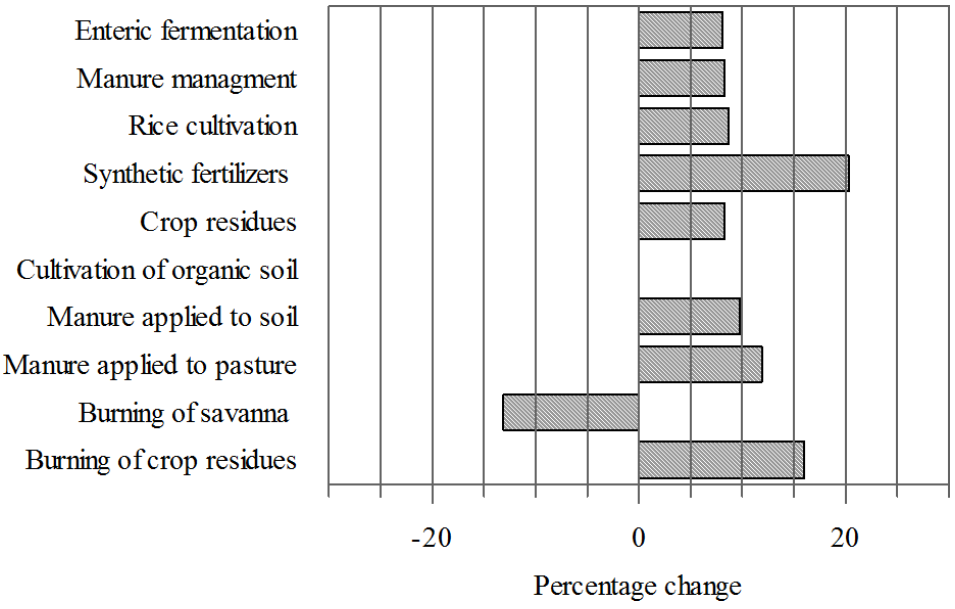

Figure 1. Percentage change in greenhouse gas emissions by agricultural source from 2004 to 2014 (data recovered from FAOSTAT [14] and modelled by the authors).

3. Plant Growth Promoting Bacteria: Plant Growth Promotion and Mechanism ofAction

Soil is a complex system where the roots draw nutrients, ensuring the growth and

development of the plants; on the other hand, the presence of insects, earthworms and microorganisms (fungi and bacteria) concur to influence the physiological and biological life of plants.

To ensure growth and productivity of crops, it is a common practice to add chemical fertilizers to quickly provide the essential nutrients to soil [17]. For the optimal growth and development of plants, herbicides, fungicides, insecticides are also added, but conversely, these compounds can damage plants and interfere with their ability to acquire some nutrients; an excessive use of these substances negatively affects human health and the environment [18,19].

Therefore, the need to use alternative systems with a lower environmental impact to ensure the best growth of plants is increasing and the use of PGPB seems to be a valid proposal.

Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella, Pseudomonas and *Serratia* are the most frequently microbial genera that have shown the ability to colonize plant rhizosphere and enhance plant growth [20].

Plants and microorganisms establish various relationships that facilitate beneficial (symbiotic and not) and/or harmful (pathogenic) interactions. During plant growth, microorganisms colonize the rhizosphere (defined by Walker *et al*. [21], as the narrow zone of soil directly surrounding the root system) and communicate with roots by producing plant growth-regulating substances. On the other hand, plants recognize microbe-derived compounds and modify their defense and growth mechanisms according to the type of microorganism [22].

These interactions occur both in phyllosphere (aerial plant surfaces) or rhizosphere (area surrounding the roots), but the environmental conditions for both sections are different. Phyllosphere is exposed to frequent changes of temperature, humidity, intensity light, etc., with consequent changes in nutrients availability. Concerning rhizosphere, it provides a better protection by changes in temperature and light intensity and guarantees a higher abundance of nutrients (more than 85% of the total organic carbon came from sloughed-off root cells and tissues or it is supply by plants as root exudates) [23]. Thus, numerous species of bacteria, fungi, protozoa and nematodes, colonize the rhizosphere, free or coated on the surface of the roots, by establishing very complexrelations.

Concerning plant-microorganisms interactions, bacteria can act as free-living bacteria, symbiotic (that form a symbiotic relationship with roots), endophytes (that colonize only a portion of interior tissue of plant having a direct access to organic compounds), and cyanobacteria (formerly called blue-green algae) [24].

An example of the beneficial interaction between plants and microorganisms is the symbiotic

interaction between the roots of legumes and some *Rhizobacteria* that lead to the formation of root nodules where the fixing of atmospheric nitrogen into ammonium, occurs [25]. *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, e *Azorhizobium* (generally known as Rhizobia) sharethesamehabitatwithPGPBandinteractthroughouttherootscolonization.Lucas-Garcia*etal*.[26], Saharan and Nehra [20] and Bhattacharyya and Jha [27] in their studies confirmed the positive effects of this cooperation showing that PGPB can facilitate the nodulation process and improve the nitrogen-fixation in the roots oflegumes.

Through direct or indirect mechanisms, or synergistically, PGPB can act on the enhancement of the performances of plants. Due to the direct action, PGPB provide the plants with some bacterial- synthesized compounds, modulate plant hormone levels that stimulate the proliferation of plant cells and facilitate the uptake of nutrients by fixing atmospheric oxygen, solubilizing minerals (such as phosphorus) and producing siderophores able to sequester iron. While, through indirect actions PGPB produce antagonistic substances or induce resistance against pathogens to prevent their harmful effects [28].

Saharan and Nehra [20], Glick [24], Bhattacharyya and Jha [27], Ahemad and Kibret [11], Beneduzi *et al*. [29] de Souza *et al*. [2], Kundan *et al*. [30] and Oteino *et al*. [31],published interesting reviews and articles on direct and indirect mechanisms; the most important findings are summarized in Table1.

PGPB can be also employed as biocontrol agents, namely organisms able to kill other pathogens or organisms causing disease to crops. *Agrobacterium, Bacillus*, *Burkholderia, Pseudomonas* and *Streptomyces* belong to this group of PGPB [20]. The biocontrol activity of PGPB against soil-borne pathogens is due to mechanisms of microbial antagonism that results in a reduction of the saprophytic growth of pathogens and, consequently, in their frequency of infection. This reduction occurs through the competition for nutrients, colonization of habitats, induction of systemic resistance (ISR) in the plant host, production of antifungal metabolites. Furthermore, due to the ability of siderophore to sequester iron, PGPB subtract nutrients for the nutrition of pathogens [20].

Pseudomonas is the most important genus of *Rhizobacteria* acting as biocontrol agents; this is ubiquitous bacteria in agricultural soils. The way it uses to act as a biocontrol agent can be summarized as follows:

- It rapidly grows *invitro*;
- It quickly uses seed and rootexudates;
- It colonizes and multiplies in the rhizosphere and in the interior of theplant;

- It produces bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances) and toxic metabolites (fenazine, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and cyclic lipopeptides[32];
- It competes with othermicroorganisms;
- It adapts to environmentalstresses.

Unfortunately, pseudomonads are unable to produce resting spores (as do many *Bacillus* spp.) and this is a challenge to produce commercial formulates [20].

Among PGPB acting as biocontrol agents, a modified strain of non-pathogenic *Agrobacterium radiobacter* K84 has been used against *Agrobacterium tumefaciens*, responsible for the collar cancer.

A. radiobacter K84 produces agrocina 84, an antibiotic compound, toxic for *A. tumefaciens*. Another application of *Agrobacterium* is due to its ability to survive and persist on the roots, which concurs to prevent the development of disease caused by pathogenic bacteria [33].

4. PGPB asBiofertilizers

Since 1990s some researchers have reported the potential of PGPB to promote plant growth and enhance the yield of crops in different soil and environment; in particular, *Azospirillum*, *Azotobacter*, *Bacillus* and *Pseudomonas* spp. were studied and applied in herbaceous crop systems as biofertilizers [34-38]. Some promising and controversial results on the mechanisms PGPB-plant root were reported [35, 36, 37, 39, 40]. There is not an official definition of biofertilizers; therefore, we refer to the definition of Vessey [41], "biofertilizer is a substance containing living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant". This definition separates biofertilizers from organic fertilizers, which contain organic compounds and directly increase soil fertility. Therefore, biofertilizers should contain living organisms, which improve nutrient use efficiency of the host plant, through different mechanisms. A keyfactor, reported by Dobbelaere *et al*. [42], is the production of growth substances such as exogenous microbial auxins and gibberellins that showed stimulatory effect on plant growth. Vessey [41] also reported an estimated N contribution by PGPB N fixing prokaryotes of ca. 1–60 Kg/ha per year, thus the biofertilizers could reduce the need for chemical fertilizers and decrease adverse environmental effects.

Five mechanisms are mainly studied as modes of action of PGPB as biofertilizers: (1)

biological N_2 fixation, (2) increasing the availability of nutrients in the rhizosphere, (3) inducing increases in root surface area, (4) enhancing other beneficial symbioses of the host, and (5) combination of thereof [24,41]. Table 2 offers an overview of the most important advances. To the best of our knowledge, no study showed the application of "autochthonous bacteria" as biofertilizer. Moreover, the benefits of PGPB were observed only when organic or chemical fertilizers were added. A depth knowledge of (1) how and which bacteria are involved in N-transformation of "the modern" agricultural system and (2) abiotic factors (pH, temperature etc.) that control nitrification and denitrification is important to gain the application of PGPB in soil asbiofertilizers.

Table 1. PGPB direct and indirect mechanisms.

DIRECTMECHANISM	MICROORGANISMS
PHOSPHATE SOLUBILIZATION Some rhizosphere colonizing bacteria enhance phosphorous availability by making soluble, insoluble phosphate inorganic compounds or by liberating organic phosphates.	Bacteria: *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Serratia* together with *Pantoea* and *Klebsiella, Rhodococcus*, *Arthrobacter*, *Chryseobacterium*, *Gordonia*, *Phyllobacterium* and *Delftia*. Rhizobia: *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum.* Fungi: *Aspergillus* and *Penicillium*.
NITROGEN FIXATION Atmospheric nitrogen (N_2) is not accessible to most living organisms but through Biological Nitrogen Fixation (BNF) it is reduced to ammonia (NH_3). BNF is performed by symbiotic or non-symbiotic nitrogen fixing microorganisms.	Non-symbiotic: *Cyanobacteria*, *Acetobacter, Achromobacter, Alcaligenes*, *Anabaena*, *Arthrobacter*, *Azoarcus*, *Azomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Corynebacterium*, *Derxia*, *Enterobacter*, *Gluconoacetobacter diazotrophicus, Herbaspirillum* sp. *Klebsiella*, *Nostoc, Pseudomonas*, *Rhodospirillum*, *RhodoPseudomonas* and *Xanthobacter*. Symbiotic: *Rhizobia*, *Frankia*.
INDOLEACETIC ACID (IAA) IAA performs many functions: (i) affects cell division, extension and differentiation of plants, stimulates the germination of seeds and tubers, (iii) exerts a control on processes of vegetative growth, (iv) concurs to increase the rate of xylem and root growth, (v) initiates formations (lateral and adventitious) of the roots, (vi) modulates responses to light, gravity and fluorescence, (vii) affects photosynthesis, pigment formation, biosynthesis of various metabolites, (viii) resists to stressfulconditions.	Several microbial species are able to produce IAA, through five biosyntetic pathways: (1) Saprophytic species of *Agrobacterium*, *Azospirillum*, some species of *Bradyrhizobium*, *Enterobacter*, *Erwinia herbicola*, *Klebsiella, Pseudomonas* and *Rhizobium* through via indole-3- pyruvic acid and indole-3-aceticaldehyde; (2) *Pseudomonads and Azospirilla* through the conversion of tryptophan into indole-3-acetic aldehyde with tryptamine as intermediatecompound; (3) *Agrobacterium tumefaciens*, *E. herbicola*, *Pseudomonas fluorescens, Pseudomonas syringae* and *Pseudomonas putida* through the formation ofindole-3-acetamide;

	(4) *Cyanobacterium* (*Synechocystis sp.*) through the formation of indole-3-acetonitrile; (5) *Azospirilla* and *Cyanobacteria* are also able to produce IAA through a tryptophan-independentpathway
SIDEROPHORE PRODUCTION The production of siderophores can be classified as indirect mechanism when they are employed as means of biocontrol by microorganisms that do not use any other mechanism. By sequestering iron, PGPBs siderophore-producing reduce the availability of this element necessary for the growth of pathogens. Siderophore mediated iron scavenging in Gram-negative transport isbetter studied PGPB than Gram-positivePGPB.	*Bradyrhizobiumjaponicum*, *Rhizobiumleguminosarum* and *Sinorhizobiummeliloti* are the main microorganisms involved in siderophore production. Among Gram-negative bacteria, siderophore-producing belong to the *Pseudomonas* and *Enterobacter* genera, while *Bacillus* and *Rhodococcus* are the most representative among Gram-positive bacteria.
ACC DEAMINASE ACTIVITY 1-aminocyclopropane-1-carboxylate (ACC) deaminase is a bacterial enzyme involved to reduce the level of ethylene in plants. PGPB in response to tryptophan or other small molecules (see IAA section) synthetize and secrete IAA. This bacterial IAA, together with endogenous plant IAA, stimulate plant growth or induce the synthesis of the plant enzyme ACC synthase which converts the compound *S*-adenosyl methionine (SAM) to ACC, the immediate precursor of ethylene.	*Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* etc.
PRODUCTION OF ANTIBIOTIC /ANTIFUNGAL METABOLITES Phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide.	*Fluorescent Pseudomonads, Azospirillum, Azotobacter, Bacillus, Enterobacter, Paenibacillus, and Streptomyces.*
COMPETITIONFORNUTRIENTS	*Actinobacteria, Azospirillum brasilense, Bacillus amyloliquefaciens,* *Bacillus pumilus, Bacillus sp., Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Brevibacillus, Enterobacter* sp., *Jeotgalibacillus, Lysinibacillus, Paenibacillus, Paenibacillus polymyxa, Pseudomonas* sp., *Pseudomonas chlororaphis, P. fluorescens, Pseudomonas aeruginosa, Terribacillus*
INDUCED SYSTEMIC RESISTANCE(ISR) "Induced resistance" is referred to the induced state of resistance in plants triggered by inducers (biological or chemical); this state protects non-exposed parts against possible attack by pathogenic microbes and herbivorousinsects.	*P. fluorescens*, *Serratia marcescens*, *Pseudomonas protegens*.

5. PGPB and Wheat with a Focus on DurumWheat

Inoculation of PGPB to enhance performance and growth is particular interestingly in wheat.

Veresoglou and Menexes [51] conducted a meta-analysis on 59 available articles focused on 228 field trials (only 12 trials on *T. turgidum* L. ssp. *durum*), and reported that *T. aestivum* ssp. *vulgare* may be a more responsive species when inoculated with *Azospirillum* spp. Moreover, the authors established linear regression models for the relationship between the effect sizes of seed yield and aboveground biomass separately for the field and pot trials. For durum wheat, the results must be confirmed. Some papers reported *Azospirillium* spp. and in particular *Az brasilense*, as one of the best PGPB able to promote growth, yield, nutrient uptake and productivity of wheat [47, 52-55].

Table 2. Application of biofertilizers in cereals.

Culture conditions	**Species/ *cultivar***	**PGPB**	**Effects on plant growth and productivity**	**References**
Field	*Triticum aestivum* L. cultivar Buck Suren(R)	*Azospirillum brasilense* and *Pseudomonas fluorescens*	Grain yield increases were not significant.	[35]
Field	*Triticum aestivum*	*Azospirillum* and *Azotobacter* sp	The inoculation increased: plant height, spike number per unit of area, grains number per spike, 1 000-grains weight, grain yield, biological yield and grain protein content.	[37]
In vitro	*Triticum aestivum* cv ProINTA	*Azospirillum brasilense* Sp245	The beneficial *Azospirillum*-wheat association is not hampered by the presence of Tebuconazole. *Azospirillum* increased root surface and promoted coleoptile length.	[43]
Field	*Triticum aestivum*	*Azospirillum brasilense*	The biofertilization reduced production costs and increased productivity.	[36]
Field	Wheat variety Zardana	*Azospirillum* and *Azotobacter* sp	The application of biofertilizer in combination with mineral fertilizer N 45kg ha^{-1} and P_2O_5 30 kg ha^{-1} increased fresh yield from11% to 59% and grain yield by 20-46%.	[39]
Field	*Triticum aestivum*	*Methylobacterium* spp.	Increased plant growth and productivity, in an environment-friendly manner.	[44]
Field	Rice	*Pseudomonas fluorescens* and *Azospirillum brasilense*	PGPB inoculation increased aerial biomass production, harvest index, and grain yield of the Supremo 13 cultivar by 4.7%, 16%, and 20.2%, respectively.	[45]
Field	Corn	*Azospirillum* and *Azotobacter* sp	Reduction in production costs with increased productivity.	[38]
Greenhouse and field	Corn	*Azospirillum brasilense*	The inoculation of *A. brasilense* had the same grain yield when compared to nitrogen treatment. The grain production was increased by 29% in the treatment with *A. brasilense* and nitrogen compared to nitrogen fertilization alone.	[46]
Field	Durum wheat (cv. Anco Marzio	*Bacillus* sp.	Soil inoculation with PGPR had a positive impact on plant growth in combination with organic fertilizer was added.	[40]

Field	*Triticum aestivum* L	*Bacillus* sp. *Stenotrophomonas* spp. *Acetobacter pasteurianusSteno trophomonas* spp	Plant growth-promoting Rhizobacteria (PGPR) provided a significant increase in shoot and root length, and shoot and root biomass. The study indicates the potential of these PGPR for enhancing growth and nutrient content of wheat and other crops under field conditions.	[47]
Pots and field	Wheat var. Inqlab-91	*Pseudomonas moraviensis* and *Bacillus cereus*	PGPR consortium with sugarcane husk and maize straw (biofertilizer formulation) increased growth, maintained osmotic balance and enhanced the activities of antioxidant enzymes and yield parameters.	[48]
Controlled conditions	Wheat	*Streptomyces* spp	These isolates can be used to design new biopesticides and biofertilizers with antibacterial and antifungal effect.	[49]
Field	Wheat, maize	*Paenibacillus polymyxa* WLY78	Nitrogen fixation, IAA production and phosphate solubilization performed by *P. polymyxa* WLY78 inside roots, stems and leaves and on root surfaces positively contributed to plant-growth promotion.	[50]

Pérez-Montano *et al*. [56] focused on the improvement of crop production by microorganisms for several cereals and leguminous and reported that in wheat, an ACC-deaminase producer *Pseudomonas fluorescens* strains concurred for the reduction of N, P and K fertilizer rates. Moreover, wheat crops resulted with higher grain yields, harvest index and protein content with lower fertilizer doses than those conventionally applied. An enhancement in grain yields was also found when two phosphate (PO_4^{3-})-solubilizing microorganisms (PSM), *Bacillus circulans* and *Cladosporium herbarum* were combined with arbuscular mycorrhizal fungi (AMF). This kind of consortia affected also the grain and soil quality and the nutrient uptake ofwheat.

There are many papers dealing with the benefits of PGPB towards legumes, maize, potatoes and wheat, but there are few reports on durum wheat. This is a crop cultivated in the Middle East, North Africa, the former USSR, the North American Great Plains, India, and Mediterranean Europe.

Durum wheat grows on 8 to 10% of all the wheat cultivated area [57]. Despite of its low acreage, durum wheat is economically important and is considered the hardiest of all wheats. Pasta is the excellent product derived from durum wheat but other products than pasta are also made from this cereal; couscous, made from durum semolina, is consumed mainly in North Africa; flat bread made from durum wheat and bulgur are consumed in Jordan, Lebanon, and Syria[57].

Depending on microorganisms, environmental and soil conditions, the interaction PGPB-durum wheat can act in different ways. Improvements in nutrient uptake (mainly N uptake), growth yield and grain quality, were reported by Saia *et al*. [40], Colla *et al*. [58], and Di Benedetto

et al.[59].

Saia *et al*. [40] inoculated PGPB and arbuscular mycorrhizal fungi (AMF), alone and in combination, in durum wheat in a field experiment in Sicily. The authors observed that the presence of AMF in soil increased plant growth and N uptake of durum wheat compared to the uninoculated control irrespective of fertilization. PGPB provided beneficial effect on plant growth and nutrient uptake only when organic fertilizer was added. The authors concluded that soil inoculation with AMF and PGPB (alone or in combination) could be an alternative way for farmers to improve nutrient uptake and the sustainability of the agro- ecosystem, although further investigation are necessary.

Colla *et al*. [58] coated seeds of durum wheat with a microbial consortium of endophytic fungi (*Glomus intraradices* BEG72, *Glomus mossae* and *Trichoderma atroviride* MUCL 45632) with the aim to enhance growth, nutrient uptake, yield and grain quality. They found that this fungal cocktail enhanced the emergence time and shoot biomass of wheat seedlings, through an increase in root dry weight. Finally, an improved grain quality, in terms of protein, P, K and Fe concentration was recovered.

As few data are available on the interaction plant-PGPB isolated from Italian soil, in a recent work, Di Benedetto *et al*. [59] started a new research focused on the selection and characterization of PGPB, from a soil of South Italy (Capitanata, Apulia region) with high potential to enhance nutrient use efficiency. Competitive strains able to survive and establish in durum wheat rhizosphere, were isolated and three strains of *Pseudomonas* spp. showed characteristics of concern for the improvement of durum wheat nitrogen use efficiency.

Furthermore, two soils differing in both texture and organic carbon content were sampled. At least about 400 isolates were collected. Odds in microbial cell number were observed in relation to the soil site. Mesophilic bacteria and actinobacteria showed the highest concentration. All the groups were tested in relation to the capacity to improve nitrogen availability and P-solubilization. In particular, some isolates of mesophilic bacteria, *Pseudomonas* spp., and actinobacteria were able to combine both nitrification and P-solubilization capacity. For the most promising strains a genetic characterization and a quantitative analysis of the parameters under study will be performed. Furthermore, the best strains will be inoculated in soil in order to test their ability to improve nutrient use efficiency in durum wheat.

Baffoni *et al*. [60] studied the interaction PGPB-durum wheat considering another point of view. They found that two bacterial strains, *Lactobacillus plantarum* SLG17 and *Bacillus amyloliquefaciens* FLN13 were able to reduce the incidence of Fusarium head blight (FHB), a

severe disease caused by different *Fusarium* species. In a field experiment, a cocktail of the two microorganisms (applied from heading until anthesis) reduced FHB index and through some PCR-DGGE analyses the authors concluded that *L. plantarum* SLG17 was present in wheat seeds and probably act as endophytic bacteria. For these reasons, *L. plantarum* SLG17 and *B. amyloliquefaciens* FLN13 have been proposed as possible promising agents for the reduction of FHB incidence.

Mnasri *et al*. [61] studied the ability of sixty-two rhizospheric and endophytic bacterial strains *in vitro* and as seed coating for the control of two strains of *Fusarium culmorum* (Fc2 and Fc3) infecting durum wheat. The authors observed that 35% and 23% of the tested strains inhibited the *in vitro* growth of both strains. Some strains were able to produce volatile compounds that inhibit the growth, the sporulation and macroconidia germination of *F. culmorum*. The sequencing of the 16S rDNA genes of the bacteria showed that they belong to the genera *Bacillus*, *Pseudomonas* and *Microbacterium*. Then, *in vitro,* five strains were selected (four assigned to *Bacillus* and one to *Pseudomonas* genera) and inoculated together with two *F. culmorum*, in durum wheat. Results showed a reduction of the percentage of infected seeds and an improved germination and seedling vigor. Under greenhouse conditions, the virulence of the fungal strains and the specificity of the bacteria/fungi interaction, influenced the effectiveness of the biocontrol of *F.culmorum*.

6. A Focus on the Nitrogen Cycle and the Possible Role ofPGPB

The core nitrogen cycle involves four reduction and two oxidation pathways. In particular, biotic Nitrogen Fixation (Figure 2) [15] involves *Azospirillum*, *Azotobacter* [24], *Pseudomonas*, *Acinetobacter* [62], *Klebsiella*, *Bradyrhizobium*, *Bacillus*, *Mesorhizobium*, *Rhizobium*[2,11].

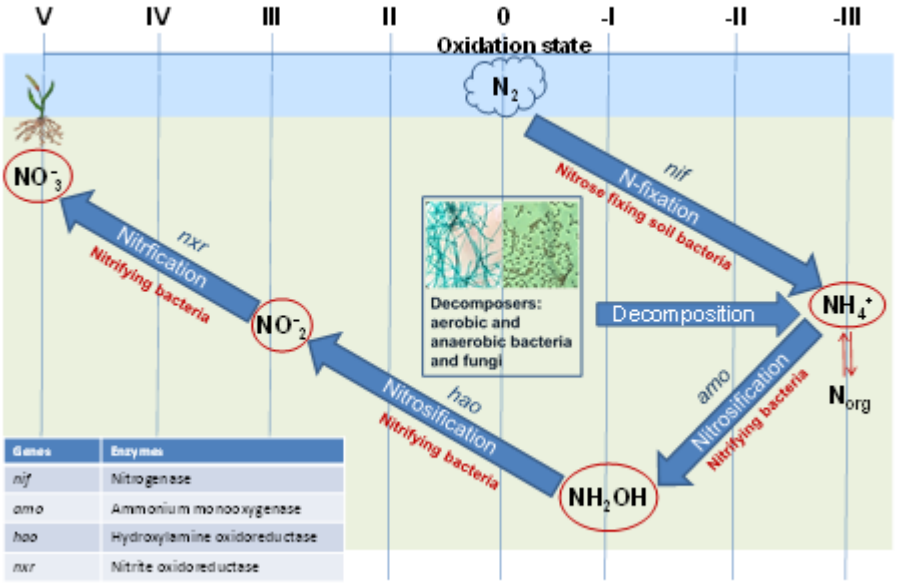

Figure 2. The major biological nitrogen pathways, that play a crucial role in transformation of fertilizing nitrogen in agricultural system (Some data were recovered by Canfield *et al*. [15]; the figure was an original work of the authors of thispaper).

Ammonia oxidation is considered to be the rate-limiting step of nitrification [63] and has received greater scientific consideration than nitrite oxidation. It is catalyzed by an ammonia oxidizing bacteria (AOB, in particular *Nitrosomonas* sp.) and ammonia-oxidizing archaea (AOA) affiliated with *Thaumarchaeota phylum* [64].

The greenhouse gas N_2O is a by-product of this process. AOB follow three distinct pathways: (i) nitrifier nitrification, (ii) nitrifier denitrification and (iii) nitrifier-coupled denitrification. During nitrifier nitrification N_2O is formed as a byproduct of the spontaneous oxidation of hydroxylamine, instead nitrifier denitrification and nitrifier-coupled denitrification are stepwise reductions controlled by enzimes during which N_2O is one of the intermediate that could escape in the atmosphere. It is not known why AOB perform nitrifier denitrification, one hypothesis is that it is a response to NO_2^- toxicity under marginally aerobic conditions [65]. Alternatively, the coupling NH_4^+ oxidization to NO_2^- reduction make nitrifier denitrification energetically favorable under marginally aerobic condition.

The last step is nitrite oxidation performed by NOB (e.g. *Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira*) [64, 66, 67]. Some studies highlighted a possible role of PGPB on both

ammonium andnitriteoxidation [11, 24]. These hypothesis were confirmed by Di Benedetto *et al*. [59]who found some wild PGPB, putatively identified as *Pseudomonas* and *Bacillus* able to oxidize ammonia to NO_2^- ions (nitrosification) and then to NO_3^- ions (nitrification).

In nitrification processes the microbial functional importance changes depending on the kind of fertilization. In literature it is reported that in an alkaline soil the increase of nitrification by chemical nitrogen fertilizers is related to a change in community abundance and structure of AOB but not AOA [68]. As an effect of increased nitrogen fertilization a rising of AOB amo A genes and a little effect on AOA community composition [69] wasobserved.

The change of edaphic factors from excessive fertilization affects the great differences of nitrifiers in physiology and metabolic pathways [64]. In particular, Nicol *et al*. [70] considered the soil pH an important determinant of bacterial diversity and community structure because it probably influences the chemical form and availability of substrates. In fact, at low pH the growth and activity of ammonia oxidizers will be inhibited due to the increase of NH_4^+ ions despite of NH_3 availability, the affinity to ammonia of AOB and AOA may drive to different growth [71].

Wang and Gu [67] reported that high salinity promoted bacteria growth but inhibits AOA; instead Erguder *et al*. [72] observed under low salinity (0.2–9 psu) and high C/N (12–25) that *archaeal amoA genes* were more abundant than *Betaproteobacteria amoA genes.*

In addition, the increases in organic matter favor AOA abundance and/or activity, while inorganic fertilizers lead to AOB and NOB dominated nitrification activity [66, 73].

7. N Uptake, N Assimilation and N Remobilization in Plants: a Focus onWheat

N is the principal component of the proteins that build cell and plant tissue. Cereals and other plant species can utilize N as NO_3^- and NH_4^+, which are the available inorganic forms of N absorbed by the roots from the soil solution [74]. In wheat, nitrogen is required to ensure photosynthetic activity, growth and grain yield and to produce grain storage proteins that have a key role in technological quality.

In wheat and rice, up to 80% of grain N content derives from leaves [75]. Most plants store nitrate in vacuoles and tolerate high ion concentrations, therefore nitrate might be also used an osmotic agent in plant [76]. Nitrate is used in various processes, including absorption, vacuole storage, xylem transport, reduction and incorporation into organic forms [77].

Nitrate assimilation is carried out mainly in the roots, being strongly dependent on the plant developmental stage and on the limitation of space for root growth [74]. In N assimilation process, nitrate is reduced to nitrite in the cytosol through the reaction catalyzed by the enzyme nitrate

reductase (NR) using NAD(P)H as electron donors. The NR enzyme is positively regulated by NO_3^- at light at the transcriptional level and is down-regulated at the post-transcriptional level by reversible phosphorylation during the dark period [77]. In hexaploid wheat, two genes encoding NADH-NR have been identified [78]. Since nitrite is highly reactive, plant cells immediately transport the nitrite from the cytosol into chloroplasts in leaves and plastids in root; in these organelles, nitrite is further reduced to NH_4^+ by nitrite reductase (NiR) [74]. NiR forms a complex with ferredoxin that provides electrons for the reduction of NO_2^- to NH_4^+ [79].

Ammonia (NH_4^+) (inorganic N) is then assimilated into amino acids glutamine and glutamate, which serve to translocate organic N (N remobilization). The two main enzymes involved are glutamine synthetase (GS) and glutamate synthase or glutamine-2-oxoglutarate amino transferase (GOGAT), The GS is considered to be a possible rate-limiting step in ammonia assimilation. The synthesized amino acids glutamine and glutamate are used as amino group donors to all the other N- containing molecules, other amino acids used for storage, transport and protein synthesis and to nucleotides used as basic molecules for RNA and DNA synthesis [80].

8. Improvement of N-uptake Efficiency by the InteractionRoot-PGPB

It is possible to identify three main characteristics/mechanisms of the rhizosphere that influence N-uptake efficiency (i) root size-morphology (ii) root N transporter system and (iii) interaction root- microorganisms such as PGPB.

It is reported that NO_3^- and NH_4^+ uptake systems may be enhanced by the interaction with arbuscular mycorrhizal fungi (AMF) [8,81], plant growth promoting bacteria (PGPB) [82], humic substances [83], allelopathic compounds such as cumarin [84] and inhibited by the increase of CO_2concentration in the atmosphere [85].

The use of plant growth promoting bacteria (PGPB) might be an alternative to increase NUE in important crops like wheat since these bacteria are able to increase root-system development and improve acquisition of nutrient including N.

NUE was evaluated through several methods (reviewed in Good *et al*. [86,87]). According to an extensively used definition, plant nitrogen use efficiency (NUE) is defined as the grain yield produced per unit of applied N fertilizer. It is an integration of N uptake efficiency (NUpE) and N utilization efficiency (NUtE) defined respectively as the capacity of plants to acquire N from the soil and the fraction of plant-acquired N to be converted to total plant biomass or grain yield [8,88,89]. NUtE is very important to NUE of crops because its improvement would result directly in more biomass andyield.

Plant roots, including those of wheat, release organic acids, sugars, exudates and other rhizodeposits, which characterize the "rhizosphere". Rhizodeposition can differ among wheat cultivars [90] leading to differences in various aspects of the rhizosphere microbial ecology [91]. In view of the increase of N-uptake it would be of interest to suppress pathogens and enhance root colonization by beneficial PGPB [8], in particular, those with the potential to enhance (a) N availability in the rhizosphere (N fixing bacteria and nitrifying bacteria), (b) root length and density (i.e IAA producer bacteria), (c) systemic plant metabolism and (d) microbial phytoprotection (i.e siderophores producer bacteria).

N availability is enhanced by microbial mineralization of organic N yielding ammonium in the rhizosphere (see Table 1). In wheat, the first effect is to attain higher N levels at flowering stage [92]. In particular, N availability for roots is improved by N fixation. Thus, the community of N fixers plays a key role for plant N nutrition [93]. In wheat and in other cereals, conversion of N_2 into NH_3 is performed by non-nodulating N-fixing bacteria. N-fixing bacteria occur naturally in soils including wheat rhizosphere [94,95], and inoculation with N fixers may enhance wheat yield [40,96,97]. Their diversity and activity are influenced by plant species [98,99] and cultivar [95,100,101,102]. Several studies proposed the inoculation of *Azospirillium* spp. as N fixers bacteria [35,37,43,52] to achieve higher yields.

Furthermore enhanced acquisition of water and mineral nutrients can be expected if the root system colonizes soil more extensively. Under *in vitro* conditions, wheat inoculation with rhizosphere bacteria may enhance root number and/or length, as well as root hair elongation [42,103]. These inoculation effects on root system architecture and biomass have been also evidenced in wheat [51,104]. These effects may be induced by the inoculation of PGPB producer of Indol Acetic Acid (IAA).

Many bacteria and fungi modify root system architecture by manipulating plant hormonal balance by producing phytohormones such as auxins [22], cytokinins [105,106] or gibberellins.

For example, the wheat bacterium *Azospirillum brasilense* Sp245 synthesized abscisic acid, and modified lateral root development in *Arabidopsis* [107]. The effects appear to take place via auxin signal transduction pathway [8]. Microorganisms also interfere with ethylene metabolism in roots modifying wheat root development [108] by a direct microbial production of ethylene [109], or a reduction in ethylene concentration in plant roots by the deamination of ethylene precursor 1- aminocyclopropane carboxylic acid [110].

Some rhizosphere bacteria might directly affect N metabolism in plants. Oil seed rape (*Brassica napus* L.) roots inoculated with *Achromobacter* strain U80417 increased net influx rates

of NO_3^-/NO_2^- [111]. Furthermore, it is known that the inoculation of *Arabidopsis* with *Phyllobacterium brassicacearum* STM196 enhanced the coding of two nitrate transporters, NRT2.5 and NRT2.6 [112]. In wheat, nitrate reductase activity of *Azospirillum brasilense* Sp245 contributed to N assimilation [104]. PGPB might also improve N-uptake by promoting plant health by inhibiting root pathogens [24].

9. Conclusion

The use of PGPB could be a frontier goal to achieve a positive effect on plants and reduce the negative impact of chemical and fertilizers on the environment.

Some strategies have been tailored, but there are few reports on wheat. This review covers the actual knowledge with a focus on the gaps and on some possible future routes for the research. To better understand the interactions PGPB-plant, depth studies on the following issues are required: (i) the isolation of "autochthonous non-pathogenic PGPB" from rhizosphere in different environments;

(i) phenotypic and genotypic characterization to select PGPB strains able to enhance nitrogen use efficiency; (iii) the inoculation of selected PGPB under controlled conditions to study the interaction microorganisms-plant. These are necessary steps before the final application in field. This review offers a perspective on what the future could demandfor.

Conflict of Interest

All authors declare no conflicts of interest in this pape

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Chapter 3

A study on a multi-step characterization and selection of Plant Growth Promoting Bacteria from durum wheat rhizosphere to improve nutrient use efficiency

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Abstract

The use of Plant Growth Promoting Bacteria is a frontier goal in soil science and microbiology; however, the selection of a Plant Growth Promoting microorganism with some desired properties is a complex process. Four-hundred seventy-four bacteria (mesophiles, spore-formers, pseudomonads, actinobacteria) were isolated and selected from durum wheat rhizosphere during the waxy stage. The isolates were preliminary characterized for phosphate solubilization, NH^{4+} production, nitrification and siderophores production; then, some quantitative analyses were done (production of IAA-indole acetic acid, NO^{2-}/NO^{3-} and P-mineralization) and used as input to select some promising isolates through a new approach based on median and quartiles.

The promising bacteria were identified by 16S sequencing; they mainly belonged to Bacillus genus (*Bacillus* spp., *B. endophyticus*, *B. simplex*/*Brevibacterium frigotolerans*), and an isolate was ascribed to the new genus *Lysinibacillus*. Three isolates of pseudomonads were identified as *Pseudomonas migulae* and *Stenotrophomonas* spp. Finally, two confirmatory experiments in a growth chamber were carried out on durum wheat plants grown under controlled conditions; some strains (e.g. 25A-*Bacillus* spp., 6P and 20P-*Stenotrophomonas* spp.) showed the best performances in terms of N-content, biomass, height of plants and might be proposed for their use in a field trial.

1 Introduction

Seven billion people are in the world (Glick, 2012) and 795 million of them are undernourished; a projection of world population of 9.1 billion and an increase of 70% of global demand for major grain crops within 2050 was reported by FAO (Food and Agriculture Organization of the United Nations) (Godfray *et al*., 2010). To meet this target, cereal production must increase substantially, while, at the same time, the environmental footprint of agriculture must strongly decrease. As reported by FAO, in term of production, wheat represents the third most important crop worldwide, following maize and rice, and provides food security to a large population share (FAO Statistical Pocketbook 2015).

The issue of production sustainability is even more acute in semi-arid and arid regions, such as Mediterranean arable lands, where drought and related biophysical factors create a fragile and unstable environment for production (Rayan *et al*., 2008). In these areas, durum wheat (*Triticum turgidum* L. subsp. *durum*) is the most extensively cultivated cereal.In the past decades, agricultural practices have focused on maximizing yields by increasing fertilization, mainly N and P fertilizations. However, an excessive use of these compounds causes leaching, pollution of water resources, gaseous emissions to the atmosphere, with irreparable consequences to the environment and human health (Zahid *et al*., 2015). Nitrogen is greatly susceptible to soil loss (by leaching or denitrification) and less than 50% of the supplied amount is taken up by the crop (Dyer *et al*., 2009; Gan *et al*., 2014).

Soluble inorganic phosphorus, used as a chemical fertilizer, is immobilized soon after it is applied, becoming unavailable to plants. Typically, the solubilization of inorganic phosphorus occurs as a consequence of the action of low molecular weight organic acids. Root exudates, including protons and organic acids, such as citrate, malate, and oxalate, are thought to assist in mobilizing P from fixed sources in the soil (van de Wiel *et al*., 2016).

Enhanced use efficiency of fertilizers would reduce the risk of the eutrophication and nitrite/nitrate pollution of waterways resulting, respectively, from phosphorus and nitrogen field run-off, as well as greenhouse gas emission (Hirel *et al*., 2011).

Present concerns on environmental sustainability of cropping systems aim the need of new agricultural engineering techniques to produce durum wheat of both sufficient quantity and quality. The use of bio-resources such as Plant Growth Promoting Bacteria (PGPB) seems to be the optimal alternative and seems to be very attractive for numerous researchers (Noumavo et al., 2016).

Agrobacterium, *Allorhizobium*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Enterobacter*, *Erwinia*, *Exiguobacterium*, *Flavobacterium*, *Mesorhizobium*, *Micrococcus*, *Providencia*, *Pseudomonas*, *Rhizobium*, *Serratia* and

Xanthomonas (Yadav *et al*., 2017; Karnwal, 2017) are the main microorganisms with plant growth promoting properties.

Rhizosphere is the zone of maximum microbial activity where PGPB can exert beneficial effects on plant growth, with direct/indirect mechanisms (Yadav *et al*., 2017; Karnwal, 2017). Numerous reviews focused on these mechanisms (Di Benedetto *et al*., 2017; Gouda *et al*., 2018; Yadav *et al*., 2017; Vejan *et al*., 2016) and how they contribute in enhancing the performances of the plants. Several soil microbial species exhibit P solubilization capacity playing an important role in the whole soil P cycle. The main P solubilization mechanisms employed by soil microorganisms include: (1) release of complexing or mineral dissolving compounds e.g. organic acid anions, siderophores, protons, hydroxyl ions, (2) liberation of extracellular enzymes (biochemical P mineralization) and (3) the release of P during substrate degradation (biological P mineralization) (Sharma et al., 2013). Importantly, "phosphate solubilization" and "mineralization" may coexist in the same bacterial strain.

In addition, due to their ability to enhance N availability in the rhizosphere (N fixing bacteria and nitrifying bacteria); root length and density (i.e IAA producer bacteria); systemic plant metabolism; microbial phytoprotection (i.e siderophores producer bacteria), PGPB may be considered a promising tool to increase also nitrogen use efficiency in staple crops like wheat (Glick, 2012).

Literature reports the use of PGPB as commercial biofertilizers that are generally allochthonous strains, which do not possess an adaptive capacity. Generally, wild strains naturally possess some mechanisms of "adaptive evolution" to win and overcome the stressful environmental conditions (Corbo *et al*., 2017).

The main goal of this paper was the selection of promising microorganisms, to be used as PGPB, for the improvement of N and P use efficiency in durum wheat. The research was performed in three phases: i) isolation and phenotypic characterization of PGPB from durum wheat rhizoshere with high potential to enhance nutrient use efficiency; ii) selection and genotypic identification of PGPB with the best performances; iii) validation on durum wheat seedlings grown in soil under controlled conditions.

2 Materials and Methods

2.1 Soil sampling

Soil samples, from rhizosphere (distance of 0-4 cm) of durum wheat, were collected by grubbing up plants (*cv* Saragolla) grown in a farm nearby University of Foggia (Apulia-Southern Italy 41°19'17"N 15°42'10"E) during the 2015 crop season. Bacterial isolation was performed on a silty-clay-loam soil having 1.3‰ total nitrogen content (Kjeldhal method), 34 ppm assimilable phosphorus (Olsen Method,

P_2O_5), 41.4 mg/Kg organic matter (Walkley-Black method), 8.1 soil pH. The microbiological analyses were carried out in duplicate during waxy stage of durum wheat life cycle.

2.2 Isolation of bacteria

Aliquots of 10 g of soil were diluted with 90 mL of a sterile saline solution (0.9 % NaCl solution), homogenized in a Stomacher bag (Seward, London, England), and blended for 1 min in a Stomacher Lab Blender 400 (Seward). Then, serial dilutions were carried out and plated onto appropriate medium to select and count Mesophilic bacteria (Plate Count Agar-PCA; 30 °C for 48 h), pseudomonads (Pseudomonas Agar Base added with Psedomonas Selective Supplement; 25 °C for 48-72 h), spore-formers (PCA, after heat-treating the dilutions at 80 °C for 10 min; the plates were incubated at 30 °C for 24 h), Actinobacteria (Bacteriological Peptone, 10 g/L; Beef Extract, 5 g/L; NaCl, 5 g/L; Glycerol, 10 g/L; Agar Technical n. 3, 20 g/L; pH, 7.00-7.20; 22-24 °C for 7-14 days), total and fecal Coliform (Violet Red Bile Agar, incubated at 37 and 44 °C for 18-24 h). All media and supplements were from Oxoid (Milan, Italy).

The measurement of pH on the homogenized product was performed twice on two different batches by using a Crison pH-meter, model 2001 (Crison Instruments, Barcelona, Spain), calibrated with two standard solutions buffered at pH=4.00 and 7.02.

From each plate, 5 to 10 colonies with different morphology were randomly selected, isolated, purified, labeled with a numeric code and stored at 4 °C.

2.3 Characterization of PGPB

All isolates were morphologically and biochemically characterized through phenotypic tests (Gram staining, catalase, oxidase, urease test, microscopic observation, spore production, motility, production of ammonium); ammonification, nitrification and urease test (Chatterjee *et al*., 2015) were performed, too. Siderophores production (Pérez-Miranda *et al*., 2007), 3 Indole Acetic Acid production (Dawwam et al., 2013), nitrate and nitrite excretion (García-Robledo *et al*., 2014) and phosphate-solubilizing (Chatterjee *et al*., 2015) were tested. The analyses were preliminary done through a screening step (qualitative approach) and then confirmed through quantitative methods. Three independent batches were used for quail-quantitative analysis. . The mean values of each experiment were used for statistical analyses (see also section 2.6).

2.3.1 Qualitative analysis

Detection of ammonium production: the ability of bacterial isolates to produce ammonium was assessed as described by Kumar et al. (2015). Bacteria were inoculated (250 µL of 24 h working cultures) in test tubes containing 5 mL of Peptone Water medium (10.0 g peptone; 5.0 g NaCl; 1000 mL distilled water;

7.0 pH) (Dye, 1962). The tubes were incubated at the optimal temperatures for 48-72 h (30 °C for mesophilic and spore forming bacteria, 22 and 25 °C for actinobacteria and pseudomonads). The accumulation of ammonia was detected by the addition of 0.5 mL of Nessler's reagent to each tube. A tenuous yellow or a deep yellow to brownish color indicated a small (+) or a high (++) production of ammonium, respectively.

Siderophores production: Bacterial cultures were inoculated onto Chrome Azurol S (CAS) agar, and incubated for 24 h at their optimal temperatures. CAS agar was prepared according to Pérez-Miranda et al. (2007) as follows (referred to 1 liter): CAS 60.5 mg, hexadecyltrimetyl ammonium bromide (HDTMA) 72.9 mg, Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) 30.24 g, and 1 mM $FeCl_3 \bullet 6H_2O$ in 10 mL of 10 mM HCl. Agarose was (0.9%, w/v) used as gelling agent. An indication of siderophore production was the changed color from blue to purple (as described in the traditional CAS assay for siderophores of the catechol type) or from blue to orange (as reported for microorganisms that produce hydroxamates) halo around the colonies.

Nitrates and Nitrites production (modified by Chatterjee *et al*., 2015): The nitrifying bacteria were enumerated on Winogradsky's medium containing $(NH_4)_2SO_4$ (0.5 g/L), glucose (10 g/L), Na_2HPO_4 (13.5 g/L), KH_2PO_4 (0.7 g/L), $MgSO_4$ (0.0479 g/L) $NaHCO_3$ (0.5 g/L) $FeCl_3 \bullet 6H_2O$ (0.014 g/L), $CaCl_2 \bullet 2H_2O$ (0.18 g/L), agar (15 g/L). The colonies were visualized (pink color) by flooding the plates with sulphanillic acid reagent.

Phosphate-solubilization (modified by Dawwam *et al*., 2013): Inorganic phosphate solubilizing bacteria were analyzed on Pikovasky medium containing yeast extract (0.50 g/L), dextrose (10.0 g/L), $Ca_3(PO_4)_2$ (5.0 g/L), $(NH_4)_2SO_4$ (0.5 g/L), KCl (0.2 g/L), $MgSO_4$ (0.01 g/L), agar (15 g/L).For each isolate (ca. 8 log CFU/mL), 200 µl of inoculum were poured into wells (9 mm diameter) previously cut with a sterilized cork borer into the agar medium and incubated at their optimal temperatures. A clear zone around colonies shown the phosphate solubilizing ability of isolates. The halo was measured in three directions and the average was calculated. The analyses were carried out in triplicate.

2.3.2 Quantitative analysis

Indole Acetic Acid: the production was assayed through a colorimetric method described by Dawwam *et al*. (2013). For each isolate, 250 µl of cell suspension, were inoculated in 5 mL of nutrient broth with tryptophan (0.1 g/l) and incubated at 30°C for 7 days. The cultures were centrifuged (10000 rpm for 10 min), then 500 µl of supernatant were added with 1 mL Salkowski's reagent (Dawwam *et al*., 2013) and a drop of orthophosphoric acid (85%), and incubated at room temperature for 15 min until pink (an indicator of indole production). The quantity of indole was measured at 530 nm using a microplate reader

CLARIOstar (BMG Labtech, Ortenberg Germany). A standard solution of pure indole-3-acetic acid was used to build a calibration curve (1-25 µg/mL pure IAA).

Sequential determination of NO_2^- and NO_3^-: A 2-step protocol was used according to García-Robledo *et al*. (2014).

Step 1: Nitrite determination

250 µL of each cell suspension (10^8 CFU/mL) were inoculated in 5 mL of Nutrient broth (Oxoid, Milan, Italy) and opportunely incubated. The cultures were centrifuged (10000 rpm for 10 min), then 1 mL of supernatant was transferred into 1.5 mL eppendorf vials and Griess-reagent (50 µL) was added and gently mixed. The vials were incubated at ~25 °C for 20 min; then, 250 µL of this solution (sample + reagents) was transferred into 96-well flat bottom polystyrene microplates. Absorbance was measured at 540 nm. A calibration curve was built with NO_2^- standards.

Griess reagent was prepared as described in Garcia-Robledo et al. (2014). It was composed of N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) and Vanadium (III) Chloride (VCl_3). 0.5 g of NED (N-(1-naphthyl)-ethylenediamine dihydrochloride) were dissolved in 500 mL of pure water (MilliQ). Vanadium (III) chloride (VCl_3) reagent 2% (w/v) was prepared in a 6N HCl solution (VCl_3reagent). Both reagents were mixed in equal proportions just prior to performing the analysis.

Step 2: Nitrate determination

650 µL of the sample + 65 µL of VCl_3 were gently mixed into vials and incubated at 60°C for 25 min, in a temperature-controlled bath; the vials were closed to prevent evaporation. Then, the vials were cooled and 250 µL of each sample were transferred into 96-well microplates. The absorbance was measured at 540 nm. A calibration curve was built with NO_3^- standards.

Phoshate mineralization for alkaline substrate: The sodium bicarbonate ($NaHCO_3$) procedure of Olsen et al. (1982), considered as a suitable index of P "availability", was adapted to microbial culture (Sungthongwises *et al*., 2014).

The phosphate solubilizing (PS) activity of the isolates was determined by using a reagent (A) prepared as follow: 12 g ammonium heptamolybdate $(NH_4)6Mo7O_2.4H_2O$ was dissolved in 250 mL MilliQ water and 0.2908 g antimony potassium tartrate ($KSbO.C_4H_4O_6$) in 100 mL MilliQ water. Then the two reagents were mixed into a 2 L flask. Moreover, 1 l of 5N H_2SO_4 was slowly added to the mixture and brought to 2-L volume (using MilliQ water). The reagent A was stored in a dark pyrex bottle, cool place. 250 µL of each bacterial culture (10^8 CFU/mL) were inoculated in 5 mL of Nutrient broth (Oxoid, Milan, Italy) opportunely incubated. The cultures were centrifuged (10000 rpm for 10 min); then, 250 µL of supernatant were transferred into 5 mL of $NaHCO_3$ 0.5 M, mixed and incubated at ambient temperature

(~25 °C) for 30 min. 500 µL of each solution were pipetted into a 2 mL eppendorf. Sulfuric acid (H_2SO_4 5N) was added to each sample, to reach a pH 5, p-nitrophenol indicator was also used for the acidification. Then 400 µL of the reagent B were prepared by dissolving 1.056 g L-Ascorbic acid ($C_6H_8O_6$) in 200 mL of reagent A, mixed well and brought to 2 mL volume. This reagent should be prepared as required because it does not keep for more than 24 hours.

Parallel analysis of a set (3-11 µM) of KH_2PO_4 standards were performed simultaneously for the determination of potassium dihydrogen phosphate concentrations. The absorbance of blank, standards and samples were read after 20 min into a micro-plate reader CLARIOstar BMG Labtech at 882 nm.

2.4 Selection and identification of isolates

The isolates selected after the quantitative experiments were identified by sequencing the 16S rDNA. DNA was extracted using the NucleoSpin® 8 Food kit from MACHEREY-NAGEL GmbH according to the manufacturer's manual. The amplification was done in a volume of 12 µL, comprising 2 µL of DNA and 10 µL of reaction mixture. The primers16S univ 27F (5'-3': AGAGTTTGATCMTGGCTCAG) and 16S univ 1492R (GGTTACCTTGTTGTTACGACTT) (http://themicrobiome.com/en/16s/16s-primers#.Wwam0GdG270:) (MWG Synthesis GmbH) were used at 5 pmol. The PCR program was set with an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation (95 °C for 15 s for each cycle), annealing (55 °C for 15 s), and extension (72 °C for 10 s)(GeneAmp™ PCR System 9700, Applied Biosystems).

The purification of PCR products was done in a volume of 11 µL (1 µL of DNA and primers 27F and 1492R at 5 pmol) through the ExoSAP-IT™ PCR Product Cleanup Reagent from ThermoFisher Scientific Inc in a BigDye™ Terminator v3.1 Cycle Sequencing Kit. PCR program included an initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 96 °C for 15 s, annealing at 55 °C for 5 s, and extension at 60 °C for 4 min (GeneAmp™ PCR System 9700, Applied Biosystems).

The products were analyzed on an Applied Biosystems™ 3730 DNA Analyzer (ThermoFisher Scientific Inc.) with Data Collection 4.0 using the POP7 polymer mix and 50 cm Array by the sequencing department of Eurofins Genomics GmbH. The assembled sequences were compared with the sequences available in the GenBank database.

2.5 Growth Chamber

2.5.1 Preparation of bacterial inocula

The isolates 12A, 25A, 36M, 40M, 97M, 6P, 20P and 23P were grown up to the stationary phase (10^8 CFU/mL) in Nutrient broth (Oxoid, Milan, Italy) and incubated at their optimal temperatures. Cell cultures were centrifuged at 4000 *g* for 5 min, and washed twice with sterile phosphate buffer (1.24 g

K_2HPO_4, 0.39 g KH_2PO_4 and 8.80 g NaCl per liter); the supernatant was discarded, and the pellet was re-suspended in sterile phosphate buffer. The viable count of the suspension was ca. 10^8 CFU/mL. The inoculated buffer was used to inoculate *Triticum durum* seeds as reported in the following sections.

2.5.2 Preparation of seeds

Sterilization of *Triticum durum* seeds (*cultivar* Saragolla, durum wheat *cultivar*, characterized by high nitrogen use efficiency) was performed by a dipping in 70% ethanol for 3 min, and a second dipping in a 3% hypochlorite solution for 10 min. Then, the seeds were washed with sterile distilled water and and germinated on moist sterile filter paper in petri dishes for 48 h.

Five seeds were treated with 1 mL of pre-inoculated buffer for 1h; seeds in un-inoculated sterile buffer represent the control (Dobbeleare *et al*., 1999). The seeds inoculated and un-inoculated were sown in pots under controlled condition (**Table 1**).Two independent experiments were done; the first one was done with the strains 12A, 25A, 36M, 40M, 97M, 6P, 20P and 23P, while a second confirmatory assay was done with the strains 6P and 20P.

Table 1: Growth chamber and physical and chemical properties of the soil used for the experiment.

Growth chamber cycle					
Days	**Photoperiod (time 24h)**		**Temperature**		**Umidity %**
			Day	**night**	**Day night**
1-9	-		6 °C	6°C	65%
10-19	7.00	17.00	10°C	8°C	65%
20-29	6.00	18.30	15 °C	10°C	65%
30-39	6.00	20.00	18°C	12°C	65%
Soil properties					
Sand		%		23.1	
Silt		%		56.3	
Clay		%		20.5	
Organic carbon†		g/kg		1.6	
Available P‡		mg/kg		54.9	
Total N§		‰		1.1	
NO^{3-}		mg/kg		15.5	
Soil pH				8.2	

†Walkley-black method; ‡Olsen method; §Kjeldhal method

2.5.3 Growth Chamber assay

Saragolla was grown in soil under controlled conditions of temperature, humidity and photoperiod (the growth chamber cycle as well as the physical and chemical properties of the soil were reported in **Table 1**) in pots (15x15x20, 3.6L, 0.0225 m^2). Nitrogen was applied as urea (50 kg/ha), 50% as base fertilization

and 50% as cover fertilization. Phosphorous was supplied at sowing as triple superphosphate P_2O_5 (45 Kg/ha). Irrigation was performed to keep optimal soil moisture (80% of available water) for wheat growth (field capacity 32%; wilting point 15%).

For each experiment, a randomized design was used with three biological replications. In the first experiment sowing was performed on 21 September 2017 and harvest on 13 November 2017 at tillering. As for the second experiment, sowing was performed on 10 November 2018 and harvest on 31 January 2019, at tillering. Plant aerial height was determined by excluding roots and biomass dry matter was determined after drying in a forced-air oven at 60 °C for 48 h. Nitrogen concentration was analyzed through Dumas method (Leco model FB-428) and expressed as reported by Khayat et al., 2006. N content was calculated by multiplying the N% by dry weight.Microbiological analyses and determination of pH of the soil, were performed during the growth cycle, as reported above.

2.6 Statistics

All the quali-quantitative analyses were carried out on three independent batches. For each experiment two technical replications were performed. The mean values of each experiment were used for statistical analyses.

The results of the qualitative analyses (siderophores, nitrite and nitrate, phosphate solubilisation, NH_4^+ production, section 2.3.1) were converted in numeric codes (0, negative in all replicates of each isolate or positive only in a replicate; 1, positive in all the replicates) and used as input values to run a Principal Component Analysis; the Euclidean distance was used as the amalgamation method.

The results from the quantitative analyses (section 2.3.2) were analysed by the coefficient of variation (CV), median and quartiles. First, the replicates for each parameter and each isolate were analysed by means of CV; if CV was >10%, the outlier test was done to remove the replicate different from the other two. Then, the median and the quartiles of each parameter were evaluated by using all replicates of all isolates and the quantitative data of each isolate were converted in a qualitative variables with four possible levels (0, 1, 2, 3), as reported in **Table 2.**

Table 2: Qualitative codes for IAA production, P-mineralization and nitrification. X, mean values of the assayed parameter for each isolate.

Code	X-value
0	Negative to the assay
1	$X \leq$ median
2	Median$< X \leq 3^{rd}$quartile
3	$X > 3^{rd}$ quartile

The results of the growth chamber were analyzed through a one-way ANOVA; the test of Tukey was used as the post-hoc comparison test and P-level was set to 0.01.

The statistical analyses were done through the software Statistica for Windows, ver. 12.0 (Statsoft, Tulsa, Okhla.).

3 Results

3.1 Qualitative screening

In the rhizosphere, mesophilic and spore-forming bacteria showed the highest cell number (ca. 7 log CFU/g), whereas actinobacteria and pseudomonads were at lower levels (ca. 6 log CFU/g). Coliforms were always below the detection limit (ca. 2 log CFU/g); pH was alkaline (about 7.8).

Table 3 reports the results for the preliminary characterization of the isolates. As expected the spore-forming bacteria and pseudomonads were Gram-positive and Gram-negative, respectively; in addition, Gram-positive bacteria represented the most of population of presumptive mesophiles and actinobacteria. A positive response to oxidase was mainly found among pseudomonads, as one could expect from their oxidative metabolism; on the other hand, the output was isolate-dependent for the other groups. Concerning the response to H_2O_2, the isolates were mainly catalase-positive, and this trait suggested their aerobic or aero-tolerant metabolism. Other traits assessed throughout the screening were urease production and the motility at the optimal temperature of growth. Concerning urease, all spore-forming bacteria were negative to this assay; moreover, it was only found in few isolates of the other groups. The target microorganisms were also negative to motility test, as it was found in a low number of isolates (from 6% for actinobacteria to 30% for pseudomonads).

After this preliminary characterization, the isolates were also studied in relation to some traits to select promising PGPB (P-solubilization, siderophores, NH_4^+ production and nitrification). The outputs for these assays were isolate-dependent. Concerning P-solubilization, the number of positive isolates was from 30% (actinobacteria) to 65% (pseudomonads), whereas mesophiles and spore-forming bacteria showed an intermediate trend (positive isolates at 41-54%). A similar trend (the highest number of positive isolates in pseudomonads spp. 82% and the lowest for actinobacteria 30% with mesophiles and spore-forming bacteria at 49-68%) was recovered for siderophores.

NH_4^+ production was found for all pseudomonads (99%), with few exceptions, and in a low number of isolates of actinobacteria (11%), whereas the outputs were variable for mesophiles and spore-forming bacteria, as this trait was found in 50% of mesophiles and 68% of spore-formers. Finally, the 4 groups were generally negative to nitrification, as this property was only found in 13-25% of the isolates.

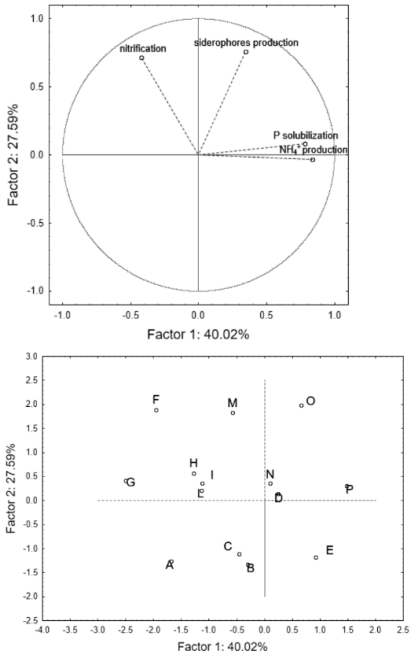
Table 3: Preliminary characterization and qualitative assays

Bacteria	**N. of isolates**	**Gram positive (%)**	**Oxidase (%)**	**Catalase (%)**	**Urease (%)**	**Motility (%)**	**P-solubilization (%)**	**Siderophores (%)**	**NH_4^+ (%)**	**Nitrification (%)**
Mesophilic bacteria	133	89	30	90	6	9	41	68	68	25
Spore-forming	96	100	39	96	0	11	54	49	50	25
Pseudomonads	65	0	80	100	1	30	65	82	99	13
Actinobacteria	180	87	52	97	10	6	32	30	11	16
Number of assayed isolates	**474**									

The selection of a promising microorganism is a kind of a risk-benefit analysis and requires a focus at isolate-level, to analyze the outputs of each microorganism and select the most promising ones. At this scope, a multivariate analysis (Principal Component Analysis, PCA) was used as a tool to gain an insight into the complexity of the four sub-population and reduce the number of the isolates for the second step (quantitative assessment of some selected traits).

Figure 1 shows variable and case distribution for mesophilic bacteria; the analysis accounted for 67% of the total variance. The first factor was positively related to NH_4^+ production and P-solubilization (correlation coefficients of 0.834 and 0.782, respectively), while the second factor was positively related to nitrification (0.717) and siderophores' production (0.762). The isolates could be divided in some homogeneous groups, as a function of the qualitative response to the different tests. In the quadrants I and IV there are the isolates positive to P-solubilization and NH_4^+production, whereas the microorganisms positive to siderophore and nitrification are in the quadrants I and II. The isolates in the group O (9M, 23M, 36M, 50M, 59M, 61M, and 77M) were positive to all the tests; on the other hand, the group A was composed by isolates negative to all the assays (5M, 7M, 8M, 25M, 28M, 39M, 53M, 65M, 99M, 106M). The other groups were characterized by responses positive/negative to the different assays.

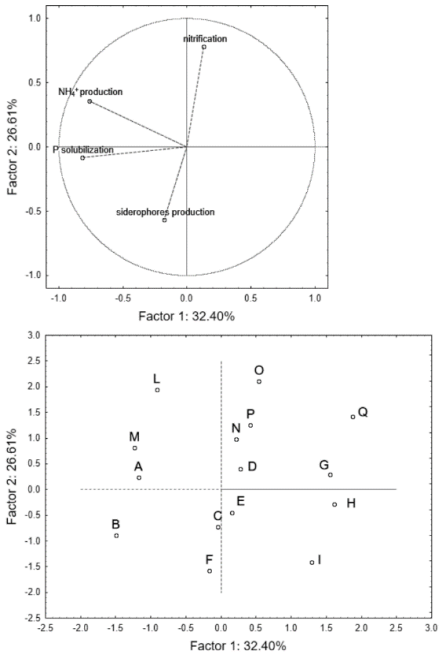
The PCA for spore-forming bacteria (**Figure 2**) accounted ca. 59% of the total variability; P-solubilization and NH^{4+} production showed a negative correlation with the factor 1 (coefficients of -0.762 and -0.816), whereas nitrification and siderophores' production were respectively related to factor 2 with a positive (0.780) and a negative (-0.568) coefficient. The group H included microorganisms negative to all assays (1B, 11B, 46B, 51B, 62B, 63B, 66B, 70B, 75B, 91B), whereas the isolates positive to all assays are in the group M (22B, 26B, 35B). Pseudomonads were positive to at least one trait and were divided in 7 phenotypic groups (**Figure 3**), but only the isolates of the group C (19P, 20P, and 30P) were positive to all assays. Actinobacteria showed a high level of complexity with 15 different phenotypic groups (**Figure 4**).



	Isolates	1	2	3	4
A	5M, 7M, 8M, 25M, 28M, 39M, 53M, 65M, 99M, 106M	-	-	-	-
B	12M, 66M, 81M, 85M, 94M, 95M, 105M, 116M, 122M, 124M, 126M	+	-	-	-
C	26M, 88M, 118M, 120M	-	-	+	-
D	1M, 6M, 24M, 31M, 33M, 37M, 71M, 98M, 111M, 130M	+	-	-	+
E	18M, 22M, 27M, 48M, 67M, 100M, 109M, 115M, 119M, 125M, 127M, 131M, 133M	+	-	+	-
F	2M, 3M, 38M, 45M, 54M, 58M, 60M	-	+	-	+
G	13M, 14M, 80M, 93M	-	+	-	-
H	55M, 79M	-	+	+	-
I	15M	+	+	-	-
L	34M, 70M, 44M, 75M, 76M, 91M, 92M, 96M, 114M, 123M	-	-	-	+
M	31M, 10M	+	+	-	+
N	78M, 128MM	-	-	+	+
O	9M, 23M, 36M, 50M, 59M, 61M, 77M	+	+	+	+
P	4M, 11M, 16M, 17M, 19M, 20M, 21M, 29M, 30M, 35M, 40M, 41M, 42M, 43M, 56M, 57M, 63M, 72M, 73M, 74M, 83M, 84M, 86M, 87M, 97M, 113M, 117M, 121M, 129M	+	-	+	+

1, NH_4^+ production; 2, Nitrification; 3, P-solubilization; 4, siderophore production

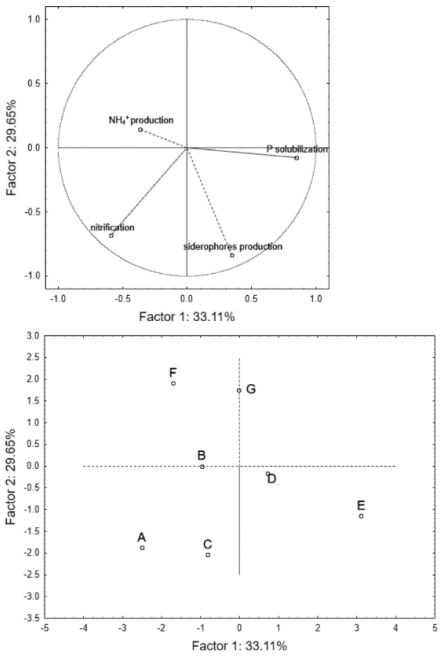
Fig. 1: Principal Component Analysis run on the output to NH_4^+ production, nitrification, P-solubilization and siderophores production for the mesophilic bacteria. The results from the independent experiments for each variable and each isolate were converted in cumulative codes, as described in section 2.6.



	Isolates	1	2	3	4
A	2B, 4B, 10B, 13B, 21B, 42B, 43B, 47B, 48B, 72B, 79B, 82B	+	-	+	-
B	5B, 17B, 7B, 27B, 30B, 37B, 45B, 53B, 54B, 58B, 68B	+	-	+	+
C	32B, 73BB	+	-	-	+
D	6B, 31B, 40B, 38B, 39B, 88B, 90B	+	-	-	-
E	12B, 14B, 28B, 33B, 44B, 49B, 50B, 56B, 87B, 92B	-	-	+	-
F	24B, 29B, 71B, 78B, 81B, 86B, 96B	-	-	+	+
G	77B, 55B, 44B, 8B	-	+	-	+
H	1B, 11B, 46B, 51B, 62B, 63B, 66B, 70B, 75B, 91B	-	-	-	-
I	89B, 20B, 36B, 18B, 23B	-	-	-	+
L	3B, 16B, 19B, 61B, 65B, 80B, 84B	+	+	+	-
M	22B, 26B, 35B	+	+	+	+
N	34B	+	+	-	+
O	60B, 93B	+	+	-	-
P	74B, 83B	-	+	+	-
Q	15B, 57B, 67B, 69B	-	+	-	-

1, NH_4^+ production; 2, Nitrification; 3, P-solubilization; 4, siderophore production

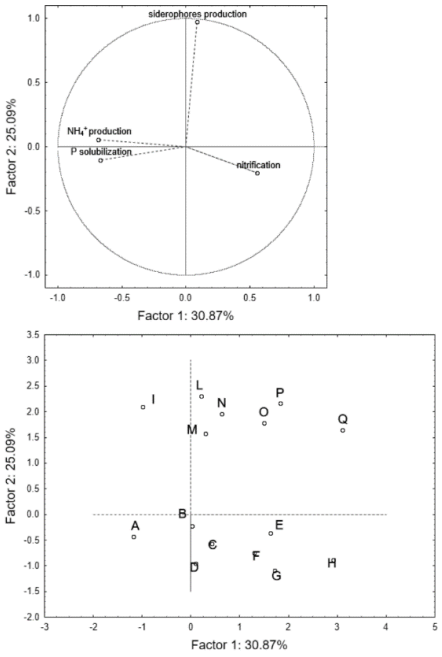
Fig. 2: Principal Component Analysis run on the output to NH_4^+ production, nitrification, P-solubilization and siderophores production for the spore-forming bacteria. The results from the independent experiments for each variable and each isolate were converted in cumulative codes, as described in section 2.6.



	Isolates	1	2	3	4
A	6P, 15P, 54P, 56P	+	+	-	+
B	12P, 14P, 32P, 47P, 53P	+	-	-	+
C	19P, 20P, 30P	+	+	+	+
D	7P, 8P, 10P, 34P, 35P, 36P, 37P, 38P, 39P, 43P, 44P, 45P, 46P, 48P, 49P, 50P, 51P, 52P, 55P, 57P, 59P, 62P	+	-	+	+
E	42P	-	-	+	+
F	26P, 24P, 27P, 29P, 56P	+	-	-	-
G	3P, 4P, 60P, 61P, 63P, 64P	+	-	+	-

1, NH_4^+ production; 2, Nitrification; 3, P-solubilization; 4, siderophore production

Fig. 3: Principal Component Analysis run on the output to NH_4^+ production, nitrification, P-solubilization and siderophores production for the pseudomonads. The results from the independent experiments for each variable and each isolate were converted in cumulative codes, as described in section 2.6.



	Isolates	1	2	3	4
A	3A, 6A, 12A, 14A, 17A, 18A, 21A, 23A, 28A, 31A, 48A, 49A, 50A, 56A, 72A, 80A, 84A, 85A, 86A, 90A, 91A, 92A, 93A, 95A, 98A, 105A, 114A, 125A, 130A, 131A, 133A, 141A, 146A, 147A, 149A, 166A, 176A, 177A	+	-	+	-
B	7A, 13A, 16A, 22A, 32A, 39A, 2A, 5A, 40A, 44A, 51A, 54A, 55A, 60A, 69A, 70A, 74A, 75A, 87A, 99A, 101A, 102A, 111A, 119A, 143A, 145A, 153A, 157A, 159A, 160A, 164A, 174A	+	-	-	-
C	122A, 123A, 132A, 151A, 165A	-	-	+	-
D	62A, 71A, 100A, 117A, 156A, 158A	+	+	+	-
E	57A, 79A, 113A, 126A, 144A, 170A, 173A, 175A	-	-	-	-
F	42A, 43A, 61A, 65A, 78A, 97A, 115A, 138A	+	+	-	-
G	83A	-	+	+	-
H	10A, 116A, 136A, 142A	-	+	-	-
I	25A, 26A, 82A, 104A, 108A, 124A, 137A	+	-	+	+
L	8A, 24A, 34A, 38A, 47A, 107A, 129A, 179A	+	-	-	+
M	1A, 103A	+	+	+	+
N	109A	-	-	+	+
O	112A	+	+	-	+
P	110A, 167A	-	-	-	+
Q	148A	-	+	-	+

1, NH_4^+ production; 2, Nitrification; 3, P-solubilization; 4, siderophore production

Fig. 4: Principal Component Analysis run on the output to NH_4^+ production, nitrification, P-solubilization and siderophores production for the actinobacteria. The results from the independent experiments for each variable and each isolate were converted in cumulative codes, as described in section 2.6.

Generally, PCA suggested a high level of biodiversity and complexity, thus pointing out the need of more restrictive inclusion criteria. Therefore, the viability was chosen as the main requisite, and the

isolates showing a viability loss throughout storage were excluded; in addition, the quantitative analyses for nitrification and P-solubilization were done only for the isolates positive to the quantitative assays. IAA production was analysed for all the isolates. As a result, the number of experiments was reduced to 1/3 (from 1422-474 isolates x 3 variables- to 333- 206 isolates for IAA, 82 for P-solubilization, and 45 for nitrification).

3.2 Quantitative analyses and identification

The data from the quantitative assays were analysed by some simple descriptive indices (median and quartiles) to study the statistical distribution of each parameter within each microbial group. The results are in **Table 4**. The main criterion to select an isolate was that the quantitative index (P-mineralization, nitrification, IAA production) should be at least as high as the third quartile (coded level 3). In addition, the number of the isolates was further reduced by a second selection and by choosing the isolates with the highest level of the parameter by means of one-way ANOVA-homogeneous group approach.

Table 4: Quantitative assays. Median and quartiles.

	Phosphate mineralization (µM)	**Indole acetic acid (µg/mL)**	**Nitrification (µM)**
Mesophilic bacteria			
Median	1.81	18.86	13.13
1 quartile	1.12	5.23	1.48
3 quartile	2.61	38.91	17.51
Spore-forming bacteria			
Median	1.49	5.33	1.62
1 quartile	0.94	3.75	0.78
3 quartile	1.79	9.09	1.97
Pseudomonads			
Median	0.91	2.27	15.83
1 quartile	-	1.38	8.20
3 quartile	-	3.65	17.32
Actinobacteria			
Median	0.49	7.21	2.44
1 quartile	0.19	4.25	0.91
3 quartile	1.61	26.38	8.16

As an example, **Figure 5** reports one-way ANOVA/method of homogeneous groups for P-mineralization in mesophilic bacteria. By using the tables of the homogeneous groups, 15 isolates were selected (6 in the group of the mesophilic bacteria, 4 amongst spore-forming, 1 for pseudomonads and 4 for actinobacteria- "first selection") (see **Table 5**).

Table 5: Selected isolates. Codes (see Materials and Methods) and criteria for the choice. P, P-mineralization; IAA, indole acetic acid production; nit, nitrification.

First selection				
	P	**IAA**	**Nit**	**Why**
36M	3	1	1	Isolates with the highest level of P amongst mesophilic bacteria
40M	3	0	0	
97M	3	0	0	
54M	0	0	3	Isolates with the highest level of Nit amongst mesophilic bacteria
58M	0	0	3	
114M†	0	3	0	Highest level of IAA amongst mesophilic bacteria
3B	3	1	1	Isolate with the highest level of P amongst spore-forming bacteria
19B	1	2	3	Isolate with the highest level of Nit amongst spore-forming bacteria
45B†	0	3	0	Highest level of IAA amongst mesophilic bacteria
89B†	0	3	0	
6P	0	0	3	Isolate with the highest level of Nit amongst pseudomonads
10A	0	0	3	Isolate with the highest level of Nit amongst actinobacteria
12A	3	1	0	Isolates with the highest level of P amongst actinobacteria
25A	3	0	0	
145A	0	3	0	Isolate with the highest level of IAA amongst actinobacteria
Second selection				
50M	2	1	1	3 properties and high resistance
60M	2	1	1	3 properties and high viability and resistance
20P	0	1	2	2 properties and high viability and resistance
23P	3	3	0	2 properties at levels >3 quartiles
Excluded isolates				

†These isolates showed a low viability, thus they were excluded and not-used for the last step of the research (identification and growth chamber assay)

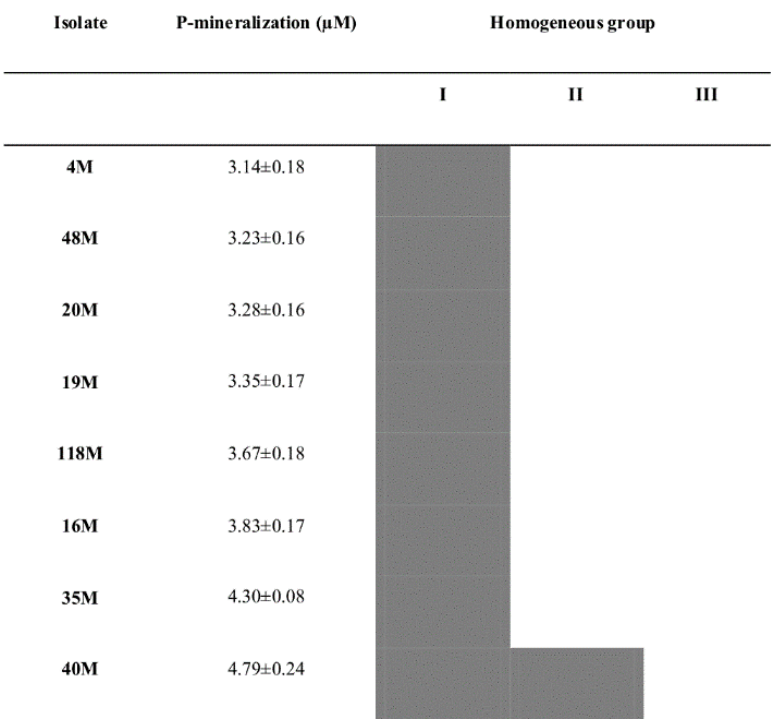


Fig. 5: One-way ANOVA (homogeneous group approach) on P-mineralization by mesophilic bacteria. Mean values±standard deviation.

In a second round, an additional criterion of inclusion was set: to choose the isolates with at least two traits amongst the assayed parameters. Therefore, other 4 isolates were selected (50M, 60M, 20P, and 23P). In the last step of the selection, the isolates 45B, 89B, and 114M were excluded because they experienced a viability loss when stored at 4°C for 3-4 weeks.

The main output of this selection was the choice of 16 isolates, which were analysed by mean of 16S-sequencing (**Table 6**).

Table 6: Identification and characteristics of the isolates selected after the second step of the research (quantitative assays).

			Qualitative analyses*			Quantitative analyses (mean±standard deviation)			
Isolate	**GenBank accession numbers**	**Identification**	**Motility**	**Sider. Production**	**NH_4^+ production**	**P-solubilization (halo, mm)**	**P- mineralization (µM)**	**Indole-3-Acetic Acid µg/mL)**	**Nitrification (µM)**
36M	*MG515459*	*Bacillus* spp.	+	+	+	1±0	4.90±0.85	0.98±0.14	0.99±0.02
40M	*MG515460*	*Bacillus* spp.	+	+	+	1±0	4.79±0.24	-**	/***
50M	*MG515469*	*Bacillus* spp.	+	+	+	1±0	2.15±0.41	0.98±0.28	1.99±0.08
54M	*MG515466*	*Bacillus simplex* *Brevibacterium frigoritolerans*	-	+	-	0±0	-	-	17.88±0.01
58M	*MG515457*	*B. simplex* *Br. frigoritolerans*	-	+	-	0±0	-	-	18.41±0.21
60M	*MG515467*	*B. simplex* *Br. frigoritolerans*	-	+	-	0±0	-	-	17.56±0.01
97M	*MG515461*	*Bacillus* spp.	+	+	+	1±0	8.90±0.44	-	-
3B	*MG515470*	*Bacillus* spp.	+	-	+	3±1	2.89±0.15	2.96±0.07	0.40±0.17
19B	*MG515471*	*Bacillus* spp.	+	-	+	4±2	1.48±0.07	5.72±0.29	7.18±0.15
6P	*MG515464*	*Stenotrophomonas tumulicola*	+	+	+	0±0	-	-	17.60±0.48
20P	*MG515465*	*Stenotrophomonas* spp.	-	+	+	2±1	-	-	15.82±0.46
23P	*MG515462*	*Pseudomonas migulae*	-	+	+	4±2	2.17±0.11	3.65±0.42	-
10A	*MG515468*	*Lysinibacillus* spp.	-	-	-	0±0	-	-	18.69±0.34
12A	*MG515472*	*Bacillus* spp.	-	-	+	2±1	8.01±0.33	5.82±0.18	/
25A	*MG515463*	*Bacillus* spp.	-	+	+	3±1	3.96±0.20	-	/
145A	*MG515458*	*Bacillus endophyticus*	-	-	+	0±0	-	140.31±7.00	/

*The result is a cumulative index from all replicates/independent batches, as reported in section 2.6
**Below the detection limit
***Not assessed

3.3 Growth chamber

In order to test the ability of the isolates to improve nutrient use efficiency *in vivo*, some selected strains were inoculated as "biofertilizers" during the growth cycle of Saragolla, a durum wheat variety well adapted to Mediterranean environment; the experiments were performed under controlled conditions of temperature, humidity and photoperiod (the growth chamber cycle as well as the physical and chemical properties of the soil were reported in **Table 2**) in pots.

The inoculation was done with 8 isolates (12A, 25A, 36M, 40M, 97M, 6P, 20P and 23P, **Table 6**), chosen among the best strains for P-solubilization (halo≥ 1 mm) and P-mineralization (40M, 97M, 12A), nitrification, ammonium and siderophore production (6P and 20P), or positive to at least to 4 tests (36M, 23P, 25A).

All isolates were used for the first experiment. At the beginning the viable count of soil was ca. 7 log CFU/g; soil inoculation determined a 1-log increase of the viable counts of the most important groups at the end of the assay; the pH was 8.2.

The results on durum wheat dry biomass and height are reported in **Figure 6** (A and B). In particular, dry matter showed the highest value for the isolate 25A, followed by the isolates 20P and 6P which showed values about 50% higher than un-inoculated control. Only an isolate (36M) was not significantly different from the control. The highest height value was found for the isolates 25A and 6P followed by the isolates 20P, 40M, 97 M and 12A. The height of the plants, inoculated with PGPB,was about 25% higher than the un-inoculated control.

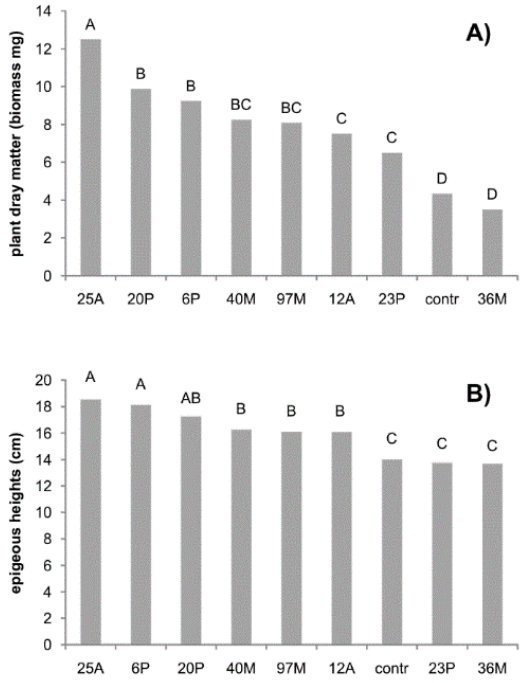


Fig. 6: Effect of selected PGPB on durum wheat biomass (A) and height (B). The letters indicate significant differences (P<0.01).

A second confirmatory experiment was done only for the strains most promising in relation to nitrogen assimilation in plants (6P and 20P); the results are reported in **Table 7**. Data confirmed the positive effect of these isolates on the dry matter and height; moreover, there was an increase of N-content in durum wheat plants whose rhizosphere had been inoculated with the two strains.

Table 7: Effect of PGPB inoculation on dry matter, height, N- content, of durum wheat plant (*cv* Saragolla) at tillering stage.

	Dry Matter (mg)	N content (mg)	N%	Height (cm)
control	24 C[§]	0.95 B	4.0[ç]	33 B
6P	30 B	1.22 A	4.1	36 A
20P	33 A	1.21 A	3.7	36 A

[§]Values in a row followed by different letters are significantly different at P≤ 0.01 (capital letters) according to Tukey's test

[ç] not significant

4. Discussion

4.1 Isolation and characterization of PGPB

The focus of this research was the isolation and the selection of autochthonous PGPB to improve nutrient use efficiency of durum wheat, a staple food with a special importance in Mediterranean area. As reported in literature the study of growth-promoting factors *in vitro* is an effective tool to select microorganisms that can be used as biofertilizers. These tests are extremely important because they allow the selection of microorganisms with better agronomic potential before testing them in plants (Szilagyi-Zecchin *et al*. 2016; Rodrigues *et al*., 2016).

PGPB are generally used as commercial biofertilizers. A critical issue is that they are allochthonous strains and could not possess an adaptive capacity; on the other hand, wild strains naturally possess some mechanisms of "adaptive evolution" to win and overcome the stressful environmental conditions (Corbo *et al*., 2017).

Different studies are reported in literature on the isolation and characterization of PGPB from rhizosphere of herbaceous crops, like *Triticum aestivum* L. (Kumaret *et al*., 2014; Majeed *et al*., 2015; Singh and Lal, 2016), pigeonpea (Ashish *et al*., 2016), rice, cabbage, sugar beet, potato (Aslam and Ahmed 2016) sugarcane (Rodrigues *et al*., 2016). To the best of our knowledge, no one investigatedthe effect of autochthonous PGPB isolated from Mediterranean environmenton *Triticum turgidum* Desf. Karadayi et al. (2016) observed a significant number of PGPB genera and isolated microorganisms belonging to *Arthrobacter*, *Bacillus*, *Streptomyces* and *Paenibacillus* spp. Other authors (Kumaret *et al*., 2014; Majeed *et al*., 2015; Rodrigues *et al*., 2016) did not observe a high bacterial biodiversity; this was probably due to the low number of bacteria isolated. In this study we isolated 474 microorganisms from the rhizosphere of durum wheat and sixteen autochthonous bacteria were selected as "potential biofertilizers".

To achieve maximum benefits in terms of fertilizer saving and better growth, many critical issues related to PGPB isolation and selection have to be solved. Criticisms concern the high number of bacterial species in soil to be analyzed; the choice of the most effective qualitative and quantitative methodologies to select PGPB as biofertilizers, and the statistics adopted for the selection and the validation method in soil of selected autochthonous bacteria.

In literature, there is not an "isolation and characterization protocol" to follow for selecting PGPB as biofertilizers and many critical issues must be addressed to obtain significant results in field.

In this paper a "more rapid" combined method of *in vitro* tests, advanced statistical analysis and *in vivo* validation, to select "the most promising microorganisms" within a large number of PGPB were put in place.

The selection of promising microorganisms is a complex process (Corbo *et al*., 2017), as there is the need to manage a large amount of data coming from many strains; in addition, each strain is characterized by many variables, with different mathematical properties (qualitative or quantitative, discrete or continuous, binary or multidimensional trends). Therefore, the scenario is a contingency table with many columns and rows.

The main challenge is to reduce the complexity of the spreadsheet but in the same time to avoid a significant loss of details/information. A second challenge is the definition of inclusion/exclusion criteria to reduce the number of the samples and select the most interesting microorganisms. A possible drawback in this step is the definition of too restrictive inclusion criteria, thus excluding interesting microorganisms, or to define decision criteria, which are not able to reduce the complexity of the contingency table.

Based on these challenges, this paper proposes an innovative selection protocol based on different steps, i.e. grouping the strains in functional classes, studying the diversity/heterogeneity inside each group, and performing a selection by using the most important variables for the final goal of this research .The dataset comprises 474 isolates; the management of a high number of isolates could lead to a bias or an under-estimation of the sources of microbial diversity. In fact, when there are many independent samples to be analyzed by multivariate analyses, there is a decrease of the accounted variability. Therefore, the phenotypic groups (mesophilic and spore-forming bacteria, pseudomonas, actinobacteria) were retained as "functional groups" for the statistical analysis.

The microorganisms from each group were morphologically and biochemically characterized to select phosphate solubilizing bacteria, nitrifying bacteria, indole acetic acid-IAA and siderophores producing bacteria (Kumaret *et al*., 2014; Majeed *et al*., 2015; Rodrigues *et al*., 2016; Singh and Lal 2016; and others). The outputs of these experiments were used to run a PCA and to evaluate the complexity/heterogeneity of the microbiota inside each functional group (second step of the selection protocol).

PCA showed a high complexity of the microbiota, with different phenotypic groups inside each class, thus suggesting the need to reduce the number of the input variables and to define criteria that are more restrictive. Moreover, the high complexity could be linked to the kind of the outputs (binary variables, with only two possibilities, negative or positive). As a result, only three variables were selected (production of IAA, NO_2^-/NO_3^- and P-mineralization), and the variables were analyzed by a quantitative protocol. The challenge for the selection is the definition of the cut-off points; the novelty of the proposed approach is the idea that a robust selection should take into account the whole population and its main

traits. Therefore, the thresholds were defined by using the data of all isolates, with a focus on quartiles and medians to reflect the effective statistical distribution of each trait. In addition, a second inclusion criteria was defined: at least two traits at the desired values to label an isolate as promising.

4.2 PGPB selection to improve N and P use efficiency

One of the main goal of this research was to study the effect of PGPB on N availability (NH_4^+ production, nitrification), as nitrogen uptake is linked to plant growth and productivity (Pii *et al*., 2015). N acquisition by roots is strictly dependent on the availability of the source itself, but about 90% of total N is present as SOM (soil organic matter). Therefore, the mineralization, i.e., ammonification and then nitrification, carried out by bacteria is crucial for plant mineral nutrition (Pii *et al*., 2015).

Concerning ammonification and nitrification, we confirmed that pseudomonads were the group with the highest capacity, being all the selected isolates able to produce NH_4^+ and NO_2^-/NO_3^-. In a previous work, Kumar et al. (2015) also found that all 75 isolates of *Pseudomonas* studied were NH_4^+ producers, although at different level (weak, moderate, or high). Moreover, we observed for *Bacillus* spp that 75% and 60% of the isolates respectively were NH_4^+ and NO_2^-/NO_3^- producers. These results are in agreement with Singh and Lal (2016), who reported the ability of *Bacillus* spp. of producing ammonia.

Phosphorous, such as nitrogen, is one of the main essential macronutrients required for plant, but 1% or less of the total phosphorus (P) in soil is considered available to plants (Raghothama and Karthikeyan, 2005; Ludueña *et al*., 2018); therefore, phosphate solubilizing bacteria are of great interest considering low P availability in agricultural soils. In fact, they can release soluble P to plants improving their growth and development (Ludueña *et al*., 2018).

In addition, the most of isolates positive to phosphate solubilization as well as to production of siderophores belonged to groups of pseudomonads (with 65% and 82% of the isolates, respectively). The species from *Pseudomonas* are generally isolated from the rhizosphere of gramineae plants; they are usually referred as "Phosphate Solubilizing Bacteria, PSB" and are involved in siderophores production (Noumavo *et al*., 2016).

Other variables used the selection of PGPB were siderophores and IAA production. Siderophores are not directly linked to growth and yield; however, many authors suggested that they could play an important role in the defense by delaying fungal growth in the root area (Numan *et al*., 2018). Indole acetic acid is an active form of auxin and an important regulator of plant growth; it plays a major role in plant growth and development (Ostrowski and Jakubowska, 2008; Vessey, 2003), by activating the radicular system (Sylvia *et al*., 2005) and increasing plant access to soil nutrients (Vessey, 2003). 68% and 82% respectively of *Bacillus* spp and pseudomonads were siderophores producers and this result highlighted

the suitability of some isolates as PGPB. Bacteria, fungi, and monocotyledonous plants in response to iron stress produce and secrete siderophores to sequester iron, in response to an iron stress (Ratledge and Dover, 2000). Microbial siderophores are classified as catecholates, hydroxamates and α-carboxylates, depending on the chemical nature of their coordination sites with iron. Pyoverdins was produced by *Pseudomonas* species and containing both hydroxamate and catecholate functional groups; *Bacillus* spp. are also known for their siderophore production (Meyer and Hornsperger, 1978; Meyer and Stintzi, 1998; Pérez-Miranda *et al*., 2007)

Relatively to the regulator of plant growth we observed that 50% and 40% respectively of *Bacillus* spp and pseudomonads were IAA producers and these data are in agreement with some literature findings (reviewed in Spaepen *et al*., 2007). Different bacterial pathways have been identified. The indole-3-acetamide (IAM) pathway is the best characterized pathway in bacteria. The IAM-related genes have been detected on the chromosome in different pseudomonads (Speapen *et al*., 2007); the tryptamine (TAM) pathway has been identified in *Bacillus* and in *Azospirillum* spp. (Spaepen *et al*., 2007).

The isolates selected in this study mainly belonged to *Bacillus* genus (*Bacillus* spp., *B. endophyticus*, *B. simplex/Brevibacteriumfrigotolerans*), to the new genus *Lysinibacillus and* three isolates of pseudomondads were identified as *Pseudomonas migulae* and *Stenotrophomonas* spp.

Bashan *et al*. (1993) reported that *Pseudomonas* and *Bacillus* are rhizosphere-associated beneficial bacteria, they antagonize pathogenic or deleterious microorganisms (biological control).

It is obvious the qualitative composition of soil microbiota, and thus of the kind of isolated bacteria, is influenced by soil conditions including temperature, moisture, and the presence of salt and other chemicals as well as by the number and types of plants found in those soils (Glick, 2012). The interaction between soil bacteria and plants may be beneficial, harmful, or neutral, therefore it is essential, after isolating and *in vitro* characterization, the evaluation of their effect in soil. As the last step of this research, a preliminary test was done with 9 isolates (12A, 25A, 36M, 40M, 97M, 6P, 20P and 23P) i.e. the best performers; then 2 isolates of these, the best nitrifying bacteria, were subsequently validated in relation to nitrogen use efficiency. The experiments were done under controlled conditions, the dry matter (a predictor of durum wheat quality and yield; Sharma, 1993), the height and the N content of the plants were determined.

Some isolates showed promising results in terms of growth enhancement (6P, 20P, 25A) which suggests the possible use of these microorganisms in a field assay.

In conclusion, the use of PGPB is a frontier goal in soil science and microbiology; however, the selection of a PGP microorganism with some desired properties is a complex process. The critical point of this

process is the management of a very complex dataset and the definition of the cut-off points and the criteria of decision. This paper reports on the selection of PGPB for durum wheat with a step-by-step approach (from isolation of several hundred microorganisms to the choice of promising targets), with a preliminary validation in a growth chamber. Further experiments are required to test the promising isolates in field trials and assess how they work in cultivated environment conditions.

5 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.

6 Author Contributions

Conceived the research plan and provided conceptual assistance: Z.F. and M.R.C; performed the experiment: N.A.D.B., D.C., M.P.C.; analyzed the data and wrote the paper: D.C., A.B., N.A.D.B.; provided conceptual assistance: A.B., M.S.

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Chapter 4

Effect of inoculation with *Stenotrophomonas* spp. on nitrogen use efficiency in durum wheat (*Triticumturgidum* L. subsp. *durum*)

Article in preparation

Abstract

The use of plant growth-promoting bacteria (PGPB) as inoculants represents a promising tool to maintain the sustainability of the cropping systems by increasing nitrogen use efficiency. In this context, the aim of the present work was to evaluate the effect of two autochthonous *Stenotrophomonas* spp. strains (6P spp. and 20P spp.) on durum wheat nitrogen use efficiency. To this aim two durum wheat genotypes were grown under controlled conditions to evaluate the capacity of the bacterial isolates to establish and survive in rhizosphere and their effect on N-uptake (UPE) and use efficiency (NUEP). Among the two investigated strains, 20P significantly increased N-uptake and use efficiency in both cultivars under study, Saragolla and Simeto. On the contrary, 6P strain did not show a positive effect on Simeto showing a different genotype response to 6P inoculation. These results suggest the potential use of 6P-*Stenotrophomonas* spp. and 20P- *Stenotrophomonas* spp. as bio-fertilizers for enhancing N content of durum wheat under low N input. Further experiments are needed to validate the effect of the application of the two selected PGPB on durum wheat NUEP and yield under field conditions.

Keywords: Plant growth promoting bacteria,*Stenotrophomonas* spp, durum wheat, Nitrogen uptake, Nitrogen use efficiency for protein.

1. Introduction

The role of soil microbes present in the plant rhizosphere has gained importance in order to improve plant nutrition and agricultural sustainability. The issue of sustainability of production is more acute in semi-arid and arid regions, such as Mediterranean arable lands. In Mediterranean area, durum wheat is the most extensively cultivated cereal. The first European producer of durum wheat is Italy (Vita *et al*., 2016) with a yield of 4.2 million tons and a surface of 1.27 million hectares. Most of durum wheat production comes from Southern Italy where Apulia Region has the largest cultivated surface in Italy (ISTAT 2018_http://agri.istat.it). Durum wheat is a staple food crop mainly used for the production of pasta, cous cous, burgul and bread. The most important grain quality characteristic for wheat is protein content that is influenced by climatic conditions, *cultivar*, nitrogen (N) fertilizer rate, time of N application, residual soil N and available moisture during grain filling (Rharrabti *et al*., 2003; Tamang *et al*., 2017). To achieve the target concentration of proteins in grains, a high level of N fertilizers is generally supplied during wheat cultivation (Henry *et al*., 2016; Tamanga *et al*., 2017). However, plants only partially assimilate the N provided, the remaining being dispersed as pollutants. Enhanced use efficiency of N fertilizers would reduce the risk of the eutrophication and nitrite/nitrate pollution of waterways and greenhouse gas emissions, which is a major concern with N fertilizers (Snyder *et al*., 2009).

Nitrogen use efficiency is defined as the grain protein per unit of available N (NUEP) and can be partitioned in N uptake efficiency (UPE, plant N per unit of available N) and N utilization efficiency (NHI, grain N per unit of N in plant) (Le Gouis *et al*., 2000; Giuliani *et al.*, 2011a). On a worldwide scale, NUE has been estimated to be as low as 33% (Raun and Johson 1999). Soil microorganisms are major players in the availability of N for plant roots in the rhizosphere (Cormier *et al*., 2016; Richardson *et al., 2009*). The use of plant growth promoting bacteria (PGPB) represents a promising tool to increase plant NUE in order to reduce the environmental pollution and N_2O emissions from N added to soil by farmers. These microorganisms may improve plant N uptake and yield by increasing root surface area and/or N availability in the rhizosphere by N fixing bacteria or nitrifying bacteria (Cormier *et al*., 2016; Richardson *et al*. 2009).

Azospirillum, Azotobacter, Nitrobacter, *Bacillus, Pseudomonas, Bradyrhizobium, Acinetobacter, Klebsiella, Mesorhizobium, Rhizobium* are the main microorganisms with plant growth promoting properties(Di Benedetto *et al*., 2017). The type and the concentration of bacteria in different soils are

influenced by the environment and soil conditions including temperature, moisture, and the presence of salt and other chemicals as well as by the number and types of plants found in soils (Glick, 2012).

Authors reported the use of PGPB as commercial bio-fertilizers that are generally allochthonous strains which could do not possess adaptive capacityto win the stressful environmental conditions at different ecological environments(Corbo *et al*., 2017). Instead, native (autochthonous) soil microorganisms naturally possess some mechanisms of "adaptive evolution" to win and overcome the stressful environmental conditions. The use of native strains as inoculants, isolated, characterized and selected for growth promoting traits of interest, could represent an important tool to search for region-specific microbial strains which can be used as bio-fertilizers. (Majeed *et al*., 2015). To date, despite some papers on the benefits of autochthonous PGPB in maize, rice and common wheat as reviewed in Pérez-Montaño *et al*., (2014), at our best knowledge less reports are available on the isolation and characterization of PGPB from durum wheat rhizosphere and their effect on nutrient use efficiency. In a previous paper carried out by our research group, a set of autochthonous PGPB was tested *in vitro* for nutrient use efficiency in durum wheat seedlings. Among them, two *Stenotrophomonas* were selected as the best strains in relation to the NO_2^-/NO_3^- and NH_4^+ production (Di Benedetto *et al*., 2019). In this study, two durum wheat genotypes were inoculated with the two selected *Stenotrophomonas* spp. isolates under controlled conditions in order to evaluate: i) the capacity to survive and establish in rizhosphere and ii) the effect on plant N uptake and use efficiency.

2. Materials and methods

2.1 Preparation of bacterial inocula

Two autochthonous strains, isolated by our research group, 6P-*Stenotrophomonas* spp. (GenBank accession no. MG515464.1) and 20P- *Stenotrophomonas* spp. (MG515465.1) were used in this experiment. As reported in Di Benedetto *et al*. (2019) 6P and 20P were characterized by the NO_2^-/NO_3^- and NH_4^+ production capacity and were selected on the basis of their promising performance in durum wheat seedlings growth.

The strains were grown up to the stationary phase (10^8 CFU/mL) in Nutrient broth (Oxoid, Milan, Italy) and incubated at their optimal temperature. Cell cultures were centrifuged and re-suspended in sterile phosphate buffer as reported by Di Benedetto *et al*., (2019). The inoculated buffer was used to inoculate durum wheat seeds as reported in the following section.

2.2 Preparation of seeds

Sterilization of durum wheat seeds (*Triticum turgidum* L. subsp *durum*, *cultivar* Saragolla and Simeto) was performed by a three step-method: a dipping in 70% ethanol (3 min); rinsing with sterile water (3 times), and treatment with a 3% hypochlorite solution (for 10 min).

Afterwards, seeds were rinsed with sterile water, dipped in sterile water for 10 min, and germinated on moist sterile filter paper in petri dishes for 48 h (Di Benedetto *et al*., 2019).

After germination, five surface sterilized wheat seeds were treated with 1 mL of pre-inoculated buffer for 1h; seeds in un-inoculated sterile buffer represented the control (Di Benedetto *et al*., 2019). The seeds inoculated and un-inoculated were sow in pots under controlled condition.

2.3 Growth Chamber assay

A completely randomized design with three factors and three replications was used. The following theses were compared, 2 N levels (0N and 50N), 2 PGPB (6P and 20P) with a control un-inoculated, 2 durum wheat *cultivar* (Saragolla and Simeto). Saragolla and Simeto were grown under controlled conditions of temperature, humidity and photoperiod, in pots (15x15x20, 3.6L, 0.0225 m^2). The soil was classified as a silty-clay soil (sand 23.1%, silt 56.3%, clay 20.5%) and characterized by 1.1‰ total nitrogen content (Kjeldhal method), 54.9 mg/kg assimilable phosphorus (Olsen Method, P_2O_5), 1.6 g/kg organic matter (Walkley-Black method) and pH 8.2.

The growth chamber cycle is reported in **Table 1**. Sowing was performed on 20 March 2017 and durum wheat was harvested on 21 June 2017. Nitrogen was applied as urea (50 kg/ha) at two different times, 50% at sowing and 50% at tillering stage. Phosphorous (P) was supplied at sowing as triple superphosphate P_2O_5 (45 Kg/ha). Irrigation brought the soil moisture to field capacity whenever the threshold of 50% available water was reached (field capacity 32%; wilting point 15%).

Anthesis occurred on 13 May 2017 for Saragolla and on 20 May 2017 for Simeto. Simeto had a grain-filling period shorter than Saragolla. Physiological maturity was achieved on 13-20 June.

Wheat yield, expressed as weight of kernels per ear, was not influenced by PGPB inoculation showing20P and 6P- inoculated plants a mean value of 403.5 mg d.m. which was not significantly different from the un-inoculated plants (384.8 mg d.m.). A significant effect of genotype ($P \leq 0.001$) was observed with Saragolla showing the higher value (421.4 mg d.m) with respect to Simeto (373.1 mg d.m.).

2.4 Soil sampling and microbiological analyses

To evaluate root colonization potential of applied bacteria, soil samples from rhizosphere were collected

during durum wheat life cycle. Microbiological analyses were carried out in duplicate to determine the number of viable cells as colony forming units (CFU) using serial dilution plating technique on agar as described in Di Benedetto *et al*. (2019). All the analyses were performed in duplicate.

Table 1: Photoperiod, temperature and humidity values during the growth chamber assay.

Days	Photoperiod (time 24h)		Temperature		Humidity %	
			Day	night	Day	night
1-9	-		6 °C	6°C	65%	
10-19	7.00	17.00	10°C	8°C	65%	
20-29	6.00	18.30	15 °C	10°C	65%	
30-39	6.00	20.00	18°C	12°C	65%	
40-49	5.00	20.00	20°C	15°C	60%	
50-90	5.00	20.30	25°C	18°C	50%	60%

2.5 Plant sampling and plant nitrogen measurements

At booting and maturity stage plant sampling were collected. Durum wheat plants were cut off at the crown and separated into leaves, culms during booting stage and into leaves, culms, chaff and grain at maturity. Plant samples were oven-dried at 65°C to constant weight to measure biomass.To determine plant total and grain N, dried plant samples were analyzed through Dumas method (Leco model FB-428) and expressed as reported by El-Khayat *et al*. (2006).

NUEP was calculated as total N grain/N fertilizer supplied to the crop and partitioned into the components UPE, total N of aboveground plant/ N fertilizer supplied to the crop and NHI, N grain/ total N aboveground plant (Giuliani *et al*., 2011a).

2.6 Statistical analysis

All data were analyzed through a tree-way analysis of variance (ANOVA) using as factors inoculum, genotype and N fertilization and the test of Tukey as the post-hoc comparison test.

3. Results

3.1 Bacterial count and survive

In **Figure 1**, soil bacterial count during the growth cycle of durum wheat plants is reported. Both 6P and 20P survived in rhizosphere soil up to 90 days after inoculation. Maximum colonization was observed at booting stage (about 45 days after inoculation). At this stage bacterial count in 6P and 20P- inoculated

pots, were a log cycle more than the total bacterial count recorded in un-inoculated control pots.

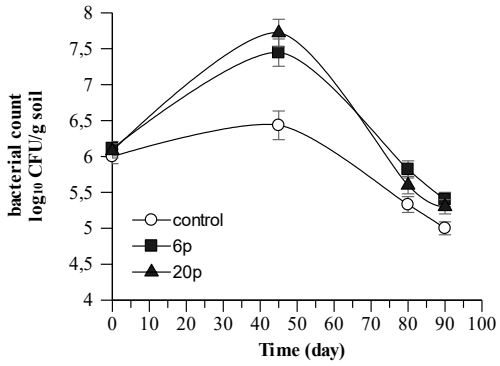

Figure 1: Soil bacterial count (Bezier curve) from sowing to harvest, expressed as log10 CFU/g soil.

3.2 Nitrogen plant content

At booting stage, a highly significant effect of PGPB inoculation, N fertilization and *cultivar* was observed for N plant content. Also, the interaction inoculum x genotype was significant ($P \leq 0.001$).

The inoculum with 20P and 6P strain determined a significantly ($P \leq 0.001$) higher plant N content (27.8 mg N and 27.4 mg N respectively), than in control plants (19.1 mg N). Also, a significant ($P \leq 0.001$) increase was observed when 50 units of N fertilizer were applied compared to unfertilized plants (26.2 mg N vs 23.3 mg N). The *cultivar* Saragolla performed better than Simeto with a mean N content of 26.7 mg N vs 22.8 mg N.

In Saragolla the highest value of N content was observed under 6P inoculum while in Simeto the best performance was observed in 20P- inoculated plants (**Figure 2**).

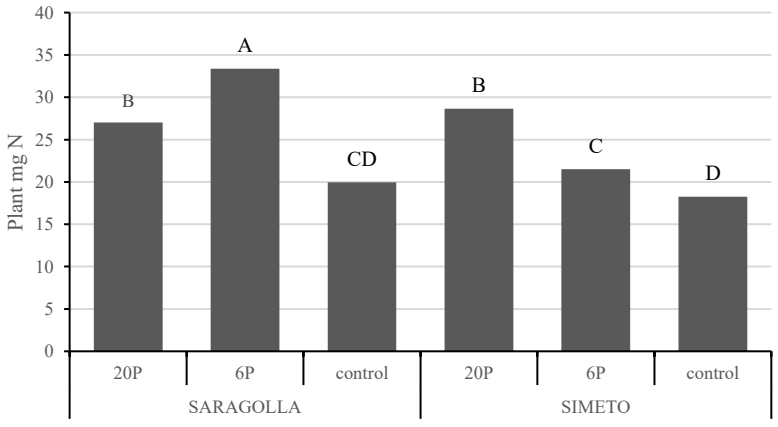

Figure 2. Effect of the interaction of bacterial inoculum with genotype on nitrogen plant content at

booting stage. Different letters indicate significant differences at P≤0.001 according to Tukey's test.

At harvest, a highly significant effect of PGPB inoculation and N fertilization was observed for N plant content and grain.

Compared to control, 20P and 6P- inoculated plants showed a significantly (P≤0.001) higher average N content in plants (35.3 mg N vs 28.8 mg N) and grains (35.8 mg N vs 29.9 mg N). The treatment with 50 units of N fertilizer produced the maximum N content (36.7 mg N) which was significantly (P≤0.001) higher than un-fertilized pots (28.4 mg N). Likewise the N content in grain in fertilized plants was significantly ((P≤0.001) higher than in unfertilized plants (35.5 mg N vs 32.1 mg N).

Also, the interaction inoculum x genotype x *cultivar* on nitrogen content in plants and kernels was highly significant (P≤0.001) (**Figure 3 A and B**). In particular, in Saragolla, both 20P and 6P strains significantly increased N content in both plants and kernels. This occurred under unfertilized and fertilized conditions. In Simeto, N content was increased only by 20P inoculum when N fertilizer was added.

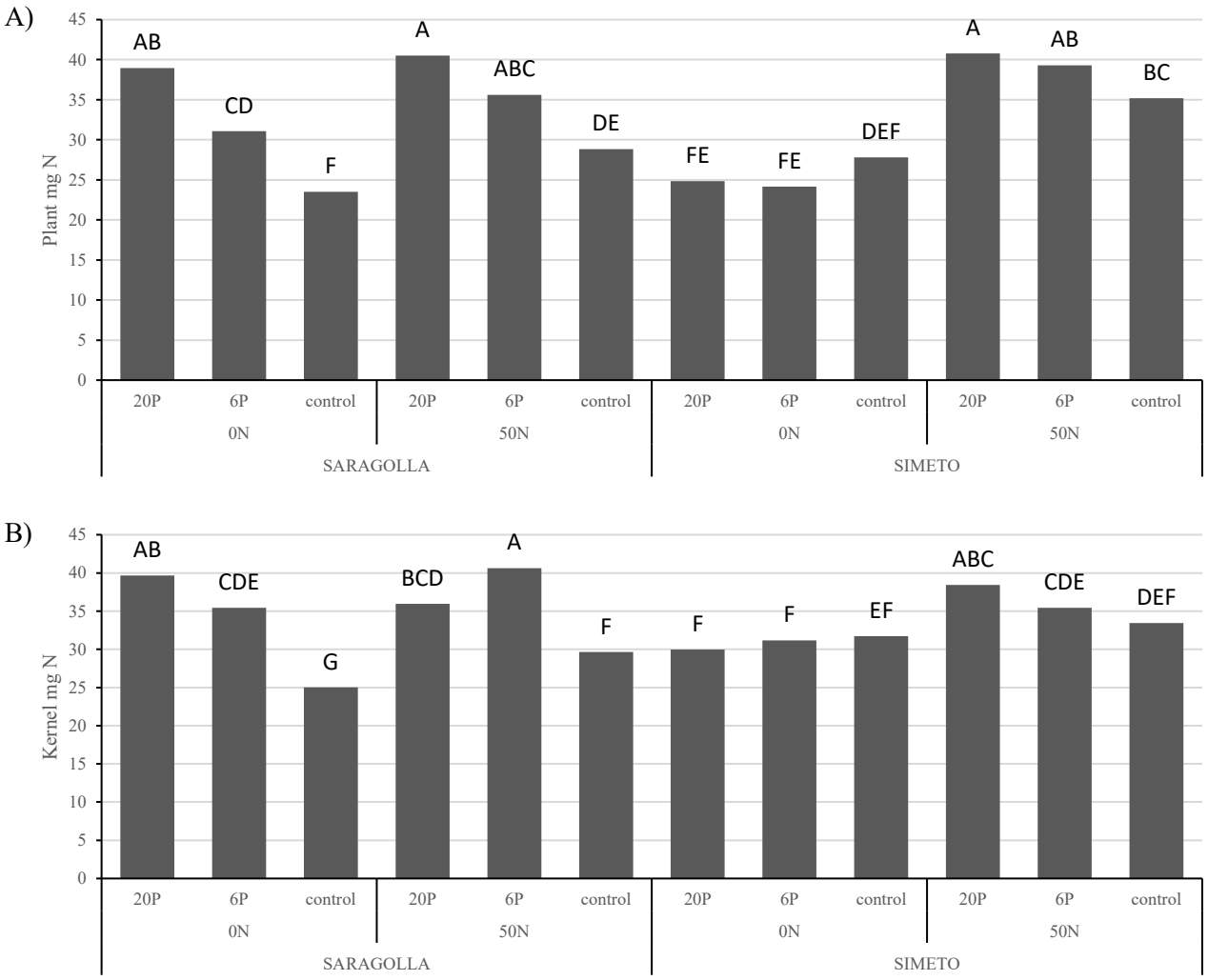

Figure 3. Effect of the interaction bacterial inoculum x genotype x N fertilization on nitrogen content at harvest, in plants A) and grains B). Different letters indicate significant differences at P≤0.001 according to Tukey's test.

3.3 Nitrogen Use efficiency and its components

Compared to control, 20P and 6P strains, determined a high significant increase of UPE and NUEP (+20% and +19%, respectively). Instead, NHI did not show differences among plants showing both control and PGPB- inoculated plants a mean value of 0.68.

The effects of the interaction between bacterial inoculum and genotype on UPE and NUPE as for 50 kg ha^{-1} fertilization is reported in **Figure 4 (A and B)**.While in Saragolla both strains significantly increased UPE and NUPE, in Simeto only 20P strain showed a significant positive effect.

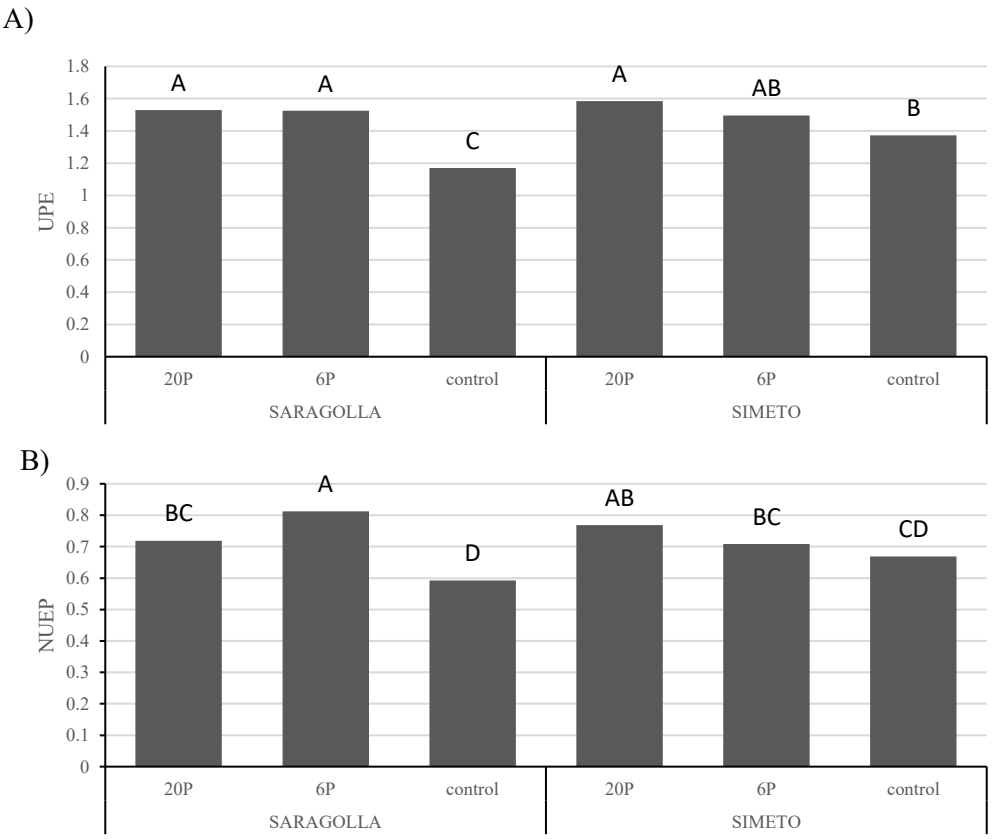

Figura 4. Effect of the interaction of bacterial inoculum with genotype on nitrogen use efficiency. A) UPE, B) NUPE. Different letters indicate significant differences at P≤0.01 according to Tukey's test.

4. Discussion

In literature, the positive effect of autochthonous PGPB is well documented in common wheat (Rana *et al*., 2012; Majeed *et al*., 2015), while, at our best knowledge less reports are available on durum wheat. In this study, a contribution to increasing knowledge on this item was given by investigating the effect of two autochthonous bacteria (6P- *Stenotrophomonas* spp. and 20P-*Stenotrophomonas*), in relation to nitrogen use efficiency in durum wheat, a species well adapted to Mediterranean conditions. 6P and 20P were able to colonize and survive in rhizosphere as observed for *Stenotrophomonas* spp. and *Bacillus* sp. in wheatby Rana *et al*. (2012) and Majeed *et al.* (2015). In particular, both strains survived up to maturity stage, showing the highest viable count at booting stage (45 days after inoculum). These results are in agreement with Majeed *et al.* (2015) who reported that the maximum*Stenotrophomonas*colonization was recorded between 30 and 45 days after inoculation. Furthermore, we observed that at this phase, soil 6P and 20P counts were, respectively, a logarithm cycle higher than control which demonstrates that in addition to the capacity to establish and survive in durum wheat rhizosphere, both strains are able to win and overcome the other bacteria present in soil.

In relation to nitrogen use efficiency, different effects of PGPB were observed during the two growth stages under investigation. At booting stage, a significant increase in plant N content ranging between 38-50% was observed in both genotypes in response to bacterial inoculation. These results are consistent with a previous experiment carried out on durum wheat seedlings (*cv* Saragolla) grown up to tillering stage under 6P and 20P- inoculation (Di Benedetto *et al*., 2019). The positive effect of autochthonous *Stenotrophomonas* isolates on N content of wheat plants (*Triticumaestivum* L. variety Inqlab–91) grown under controlled conditions was also reported in Majeed *et al*. 2015 where a significant increase in N content (+53%) was observed 50 days after *Stenotrophomonas* inoculation. Likewise, in Rana *et al*. (2012) a significant increase (up to 66%) in N content was reported in wheat plants (*Triticumaestivum* L. variety HD 2687) inoculated with N-fixing bacteria.

At maturity stage, the effects of PGPB inoculation on N-uptake changed depending on genotype. While in Saragolla both strains significantly increased UPE and NUPE, in Simeto only 20P strain showed a significant positive effect.

The positive effect of 20P and 6P on N use efficiency suggests their potential use under low N input such as the ones adopted in our experiments. These results are in agreement with Shaharoona *et al*. (2008) who carried out a study to evaluate the effect of *Pseudomonas* strains on N use efficiency in wheat (*Triticumaestivum*) under different levels of N fertilizer (30, 60, 90 and 120 kg ha^{-1}). Results of pot and field trials showed that N use efficiency significantly increased in response to inoculation at all fertilizer levels but, interestingly, the maximum NUE value was reported under the lowest N levels supplied. Despite the increase in N-uptake, no effect of PGPB inoculum was observed for NHI in both genotypes. About the influences of UPE and NHI components on N-use efficiency, contrasting results were reported in literature. As reviewed by Foulkes *et al*. (2009) for wheat plants, several authors found that under low N input, N-uptake is more important than N utilization in determining N use efficiency; consistently the relative importance of UPE decreases. Futhermore different studies reported that wheat breeding resulted in consistent improvements in harvest index than in N-uptake component. Instead other authors reported that N-uptake and utilization equally contribute to increases in NUE.

In agreement with the results reported in durum wheat Italian genotypes by Giuliani *et al*. (2011a) and Ercoli *et al*. (2013) we showed that, at low N supply (50 kg ha^{-1}), UPE was the most important component, which influenced NUPE value. On the contrary, Giambalvo*et al*. (2010) found a small correlation between NUE and UPE, and concluded that variation in UPE contributed very little to the variation in NUE.

The different behaviour of 6P strain between genotypes highlighted the importance of plant-PGPB interaction as a key factor for the success and efficiency of PGPB inoculation. Also Iniguez *et al*., 2004, evaluating the effect of *Klebsiellapneumonia* strain 342 on N accumulation in three different wheat genotypes (*Triticumaestivum*) found an increase in N content only in one genotype while the others showed nitrogen deficiency symptoms.

Similarly, Neiverth *et al*. (2014) testing the effect of *Herbaspirillum* sp. inoculation on five wheat genotypes found that two genotypes showed increased shoot and/or root weight; three genotypes were either unaffected by inoculation or responded negatively with a decrease in shoot and root weight upon inoculation.

Conclusion

N nutrition plays a key role for grain yield and quality of durum wheat with significant economic consequences for wheat producers. The use of PGPB represents a promising tool to increase NUE maintaining the sustainability of the cropping systems. Among the two investigated strains, 20P significantly increased N-uptake and use efficiency in both cultivars under study. On the contrary, 6P strain did not show a positive effect on Simeto showing a different genotype response to 6P inoculation. These results suggest the potential use of the autochthonous strains 6P and 20P as bio-fertilizers for enhancing N content of durum wheat. Moreover, further experiments are needed to validate the effect of the two PGPB on NUEP and yield of wheat plants under field conditions.

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Chapter 5

Effect of soil inoculation with Plant Growth Promoting Bacteria on plant growth of wheat seedlings submitted to phosphate starvation.

Article in preparation

Abstract

The role of soil microorganisms in the plant rhizosphere has gained importance in order to increase agricultural sustainability. The inoculation of soil with phosphorus-solubilizing (PSB) and phosphorus-mineralizing (PMB) bacteria could represent a promising strategy for the improvement of plant growth under P starvation. Certain PSB and PMB may improve plant growth by increasing root surface area due to their ability to synthesize and export indole-3-acetic acid (IAA).The aim of the present work was to evaluate the effect of soil inoculation with two autochthonous *Bacillus* sp. strains (12A- *Bacillus* sp. and 25A-*Bacillus* sp.) on wheat seedlings growth. To this aim a durum and a bread wheat genotype were grown under controlled conditions in low P soil to evaluate: i) the effect of the bacterial isolates on plant growth and root system architecture; ii) the expression of key genes involved in the P-starvation response and Pi uptake, *IPS1* and *TaPT6*. The *IPS1* gene is highly responsive to P deficiency, and its transcript level is a good marker for P deficiency responses and has been shown to be involved in P homeostasis. *Ta*PT6 is a high- affinity Pi transporter gene playing a dual role in Pi uptake from the soil and Pi translocation inside the plant.

12A- *Bacillus* sp. significantly increased root length, surface area and root biomass. Furthermore, an enhanced shoot dry weight and shoot P content was observed. These results could be explained by the capacity of 12A strain to produce indole-3-acetic acid (IAA) in addition to the P mineralizing and P solubilizing capability. On the contrary, 25A- *Bacillus* sp. (no IAA producer) did not improve root traits, plant biomass and P content in shoot maybe due to both a lower root branching since its inability to produce IAA, and to a lower capacity to mobilize unavailable P than 12A- *Bacillus* sp.

Keywords: Plant growth promoting bacteria, wheat, P-solubilizing Bacteria, P-mineralizing Bacteria, P-deficiency soil, IAA, root architecture, IPS1, PT6.

1. Introduction

Phosphorus (P) is an essential macronutrient for plants, being a structural component of nucleic acids, phospholipids and adenosine triphosphate (ATP), as a key element of metabolic and biochemical pathways, important particularly for Biological Nitrogen Fixation (BNF) and photosynthesis (de Souza *et al*. 2015). Even if P is abundant in soil in both organic and inorganic forms, it represents a limiting factor for plant growth since only a small quantity is available for root uptake. It is known that about 50% of global cultivated land suffers from P deficiency because of insufficient P replacement into agricultural systems and since much of the soluble inorganic P that is used as chemical fertilizer, is immobilized soon after it is applied becoming unavailable to plants (Heuer *et al*. 2017). In fact, when P is supplied as a fertilizer, it is rapidly immobilized in the soil because of its high reactivity with cations such as calcium and magnesium in calcareous soils or aluminium and iron in acidic soils (López-Arredondo *et al*. 2014). P deficiency severely limits crop yield, and regular fertilizer applications are required to obtain the high yields required to feed the ever-increasing world population. Recent estimates report that a global consumption of P fertilizer is increasing with an estimated 47 million tonnes of P_2O_4 by 2018 (Heuer *et al*. 2017). Despite P increasing fertilizers use, P use efficiency (PUE) has been estimated to be as low as 16% for all cereals (Dhillon *et al*. 2017) with severe consequence for the environment, not only because of diminishing P reserves, but also because an increase in the release of P from agricultural effluent lead to the eutrophication of water enhancing the critical problem of toxic algae blooms (López-Arredondo *et al*. 2014).

The central importance of Pi in plant nutrition and agricultural sustainability has long been recognized.In the past few years, considerable efforts have been made to understand how plants adapt to low-Pi stress and the mechanisms that increase Pi uptake, transport, and utilization. Plant uptake of available Pi occurs through the action of high- and low-affinity Pi transporters for Pi uptake from the soil and internal distribution, as well as for storage and remobilization of Pi from the vacuole, and is affected by root architecture and root exudation of organic acids (citrate, malate and oxalate) and enzymes (phosphatases and phytases) into the rhizosphere to solubilize inorganic P complexes and mineralize organic P respectively (Sharma *et al*. 2013; Heuer *et al*. 2017).

In addition, in the last few decades, different regulatory pathways involved in P deficiency response in plants have been described too. For example, a Pi-mediated signalling cascade, involving the Induced by Pi Starvation 1 (*IPS1*) gene, the small RNA miR399 and the genes *UBC24* and *PHO2*, is induced by P deficient soil conditions. IPS1 transcripts inhibit the action of miRNA399 silencing complexes on PHO2

mRNA (a ubiquitin E2 conjugase) thereby adjusting the transcript levels of PHO2, which is a key player in balancing Pi with respect to its supply and demand (Franco-Zorrilla *et al*. 2007; Briat *et al*. 2015; Kumar *et al*. 2017).

The role of soil microbes present in the plant rhizosphere has gained importance in order to increase Pi plant nutrition and agricultural sustainability, and also in relation to low Pi availability. The issue of sustainability of production is more acute in semi-arid and arid regions, such as Mediterranean arable lands. In the Mediterranean area, wheat is one of the most extensively cultivated cereal and provides 50% of human's dietary energy supply, together with corn and rice**(**FAO2014**)**.

The inoculation of soil with microorganisms, referred as P-solubilizing bacteria (PSB) and P-mineralizing bacteria (PMB) (Tao *et al*. 2008; Sharma *et al*. 2013), able to mobilize the poorly available P by releasing P from total soil P through solubilization or mineralization, respectively, could represent a promising strategy for the improvement of plant growth under P starvation (Singh and Kapoor 1999; Krey *et al*. 2013). The main mechanism of microbial phosphate solubilization is the production of mineral-dissolving compounds, such as organic acids, siderophores, protons, hydroxyl ions and CO_2 (Rodríguez and Fraga 1999; Sharma *et al*. 2013). Organic acids together with their carboxyl and hydroxyl ions chelate cations or reduce the pH to release P (Alori *et al*. 2017). An alternative mechanism to organic acid production for solubilization of mineral P is the release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase (Rodríguez and Fraga 1999; Sharma *et al*. 2013). Concerning the mineralization of organic P, which represents about 50-70% of total P in soil, largely stored as phytate which is not available for plant use, it is carried out by phytases produced by P mineralizing bacteria (PMB). Other enzymes produced by PMB in the process of organic P mineralization are phosphatases. The major source of phosphatase activity in soil is considered to be of microbial origin as confirmed by the presence of a significant amount of microbial phosphatase activity detected in different types of soils (Rodríguez and Fraga, 1999).

Certain PSB and PMB may improve plant growth not only by making unavailable P available for plant growth but also, by increasing root surface area since their ability to synthesize and export of organic compounds such as auxin (Spaepen *et al*. 2007). Indeed, it is reported that auxin level is usually higher in the rhizosphere, where a high percentage of rhizosphere bacteria is likely to synthesize auxins as secondary metabolites because of the rich supplies of root exudates (Spaepen *et al.* 2007).Indole acetic acid (IAA) is the most abundant endogenous auxin produced and it has been recognized as an important factor contributing to the stimulation of root development in plants (Spaepen *et al*. 2007). Given the

chemical immobilization of P in soils, root architecture is a key trait for optimizing P uptake. Only 20% of the top soil is explored by roots during plant growth, therefore enhancing topsoil foraging is essential to improving P use (López-Arredondo *et al*. 2014).

In the literature, there are some reports on plant growth promotion after inoculation of commercial PGPB that solubilize inorganic and/or organic P in soils and on their effect on root growth and the expression of key Pi transporters (Kumar *et al*. 2014; Saia *et al*. 2015; Ogut *et al*. 2016). In contrast, to our knowledge, very little is known about the expression of key genes induced by P-starvation in PGPB-inoculated plants.

Recently studies focused on the isolation, the characterization and the evaluation of autochthonous PSB and PMB which naturally possess mechanisms of "adaptive evolution" to overcome stressful environmental conditions and promote plant growth so that they can be exploited commercially as bio-fertilizers (Gupta *et al*. 2012; Sadiq *et al*. 2013; Sarker *et al*. 2014; Zhao *et al*. 2014; Manzoor *et al*. 2017). In this context, the aim of the present work was to evaluate the effect of soil inoculation with two autochthonous *Bacillus* sp. strains on growth of wheat seedlings. To this aim a durum and a bread wheat genotype were grown under controlled conditions in low-P soil to evaluate: i) the effect of the bacterial isolates on plant growth and root system architecture; ii) the expression of key genes involved in the P-starvation response and Pi uptake.

Materials and methods

1.1. Preparation of bacterial inocula

Two autochthonous P- solubilizing and P-mineralizing strains, 12A- *Bacillus* sp. (GenBank accession no. MG515472.1) and 25A- *Bacillus* sp. (GenBank accession no. MG515463.1) were used in this experiment. As reported in Di Benedetto *et al.* (2019), 12A and 25A differed for the level of P-mineralization and for the production of IAA since the 12A strain was characterized by a two fold higher P- mineralizing activity and by the IAA-production capacity.

Pure culture of the selected strains were grown up to the 10^8 cell/ml in nutrient broth (Oxoid, Milan, Italy). Cell cultures were harvested by centrifugation (4000 g, 5 min) and washed once with sterile Ringer's buffer (7.2 g NaCl, 0.17 g $CaCl_2$, 0.37 g KCl per liter, pH 7.3-7.4). The viable count of each suspension was 10^8 cell/ml. The inoculated buffer was used to inoculate soil as reported in the following sections (Liu *et al*. 2010; Dobbelaere *et al*. 1999 with adaption).

1.2. Preparation of seeds

According to Dobbelaere *et al*. 1999,*Triticum turgidum* L. subsp. *durum* (*cv*. Simeto provided by University of Foggia, Italy) and *Triticumaestivum* L. seeds (*cv*. Cadenza provided by Rothamsted Research, UK) were surface sterilized by: (i) submerging in 70% ethanol (10 min); (ii) treating with a solution of 20% NaClO (30 min); (iii) rinsing three times with sterile water. The seeds were germinated on moist filter paper in petri dishes (10 seeds/dish) at 18-22 °C.

1.3. Greenhouse experiment

7-days old seedlings were transferred into black plastic pots (11.5 diameter, 0.0132 m^2) containing one seedling per pot. The potting soil consist of autoclaved (126°C per hour) compost plus sand (Levington Advance_Seed and Modular Compost F2S). The compost, hereafter called soil, was washed to remove soluble nutrients and autoclaved again (126°C per hour). In order to ensure an adequate supply of plant-required nutrients, with the exception of P, a nutrient solution without P was added one day after potting. The nutrient solution had the following content: 5 mM KCl, 2 mM $NaNO_3$, 1 mM $MgSO_4$, 9.2µM H_3BO_3, 3.6 µM $MnCl_2$, 16 nM Na_2MoO_4, 5 µM KCl and 770 nM $ZnCl_2$ as reported in Grün *et al*. (2017) with adaption. Bacterial inoculation was performed one day after planting by adding in the soil close to seedling roots, 1 ml of bacterial inoculum. Two different bacterial inocula were tested: strain 12A- *Bacillus* sp. in Ringer's Buffer medium and strain 25A- *Bacillus* sp. in Ringer's Buffer medium at 10^8 cell/ml to each pot. Control seedlings were inoculated with the same amount of a no-PGPB (E. coli DH5α strain in Ringer's Buffer medium). The experiment was completely randomized, with four replicates per treatment, and it was carried out in a greenhouse for 20 days with a 20/15°C day/night temperature and a photoperiod of 16 h day^{-1} with natural light. Supplementary lighting was provided when light intensity fell below 100 umol and was switched off when light intensity was above 300 umol. Plants were kept well-watered using tap water.

1.4. Plant and Root sampling

Plants were destructively harvested at 20 days after inoculation (DAI). Soil was removed from the plant roots by washing with tap water and gentle brushing. The shoots were cut off from the roots and oven-dried at 80° C for 48 hours and weighted to determine the total aboveground biomass. To determine plant total P, dried shoot samples were milled and subsequently digested with HNO_3/$HClO_4$ for ICP-OES analysis.

Roots were stored in a 20% v/v ethanol solution at 4°C until morphological characterization. For the characterization, roots were placed in a 30 x 42 cm tray filled with 1 cm of tap water and scanned on a

flat- bed scanner (Epson Expression 11000XL- Epson, Japan)at a resolution of 600 dpi. The surface area and length were determined from the root images using the image analysis software WinRHIZO (*Arabidopsis* version 2013e 32 Bit- Regent Instruments, Canada), differentiating the primary and the lateral roots with a distinguishing diameter of 0.32 mm.

After scanning, roots were oven- dried at 80° C for 48 hours and weighted to determine the root dry weight.

1.5. Total RNA extraction and cDNA synthesis

Total RNA was isolated from homogenised plant material consisting of the fully expanded leaf. After harvesting, each sample was immediately snap-frozen in liquid nitrogen and stored at -80° until RNA extraction using TRizol reagent (Invitrogen Cat. No. 15596-026) (1ml TRizol/50mg plant tissue). The homogenate was centrifuged (12,000 g for 5 minutes at 4°C) and incubated at room temperature for 5 minutes;the supernatant containing the RNA was transferred to a clean tube, added of 0.2 ml of chloroform and briefly vortexed, followed by an incubation at room temperature (2 min). Each sample was centrifuged (12,000 g for 15 min at 4 °C) and 0.25 ml of isopropanol was added to the upper aqueous phase. The RNA was pelleted by centrifugation (12,000 g for 8 min at 4 °C) and then washed by applying 1 ml of 75 % ethanol and centrifugation (7,500 rpm for 5 min at 4 °C). The RNA pellet was air-dried and re-suspended in 50 µl of sterile water.

The RNA concentration was determined by measuring the absorbance at 260 and 280 nm using a NanoDrop (ND-SPECTROPHOMETER 1000, USA).

Genomic DNA was removed by DNase treatment (37°C) performed in a 10 µl reaction volume containing 8 µl of total RNA, 1 µl of DNase Buffer, 1 µl DNase. After 30 minutes, the reaction was stopped by adding one µl of Stop DNase Buffer at 67°C for 10 minutes. The integrity of the DNase-treated RNA was confirmed by electrophoresis using 1% (w/v) agarose gel. Complementary DNA was synthesised from the DNase-treated RNA using SuperScript III Reverse Transcriptase (Invitrogen) in a total reaction volume of 20 µl using 2 µg total RNA, 4 µl 5 x buffer, 1 µl 0.1 M DTT 1 µl Superscript III Reverse transcriptase as per the manufactures' instructions.

1.6. Semi-quantitative Reverse Transcriptase (RT)-PCR

To study the expression of IPS1 and *Ta*PT6 genes, Reverse-transcription (RT)-PCRs were carried out. The constitutively expressed cyclophilin gene was used as internal positive control. All primers used for the RT-PCR are listed in **Table 1.**Four replicates for each factors interaction (Genotype X Inoculum) were included in every (RT)-PCR run containing: 1 µl of cDNA solution or water (contamination control)

and 24 µl of Master Mix. Separate RT Master Mix reaction was prepared for each primer setusing the DreamTaq Green PCR Master Mix 1.1X (ThermoFisher Scientific). The total volume of the RT-PCR reactions was 25 µl. Reactions were performed as follow: 5 min at 96°C followed by 30 cycles of 96°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min.

5 µl of the RT-PCR products were analysed by electrophoresis in 1% (w/v) agarose gel stained with ethidium bromide (1µl/100ml).

Table 1. List of primers used in RT-PCR analysis.

Gene	Name	Primer sequences
IPS1	IPS1 F1	5' CGCACACCATGTAGGCCACAA 3'
	IPS1 R3	5' CTTCATATCAATTTGGTATAGCTAGCTA 3'
***Ta*PT6**	*Ta*PT6 F1	5' AATTAACCTGGACAACTCGACC 3'
	*Ta*PT6 R3	5' CTGGCGCAGAACAAGGACC 3'

1.7. Statistical analysis

Statistical analysis was performed by using the statistical package Statistica, version 7 (StatSoft, Tulsa, OK, USA). All data were analysed by two-way analysis of variance (ANOVA) using genotype and inoculum as factors. Tukey's test was used as post hoc to assess significant differences at 0.05, 0.01 and 0.001 P level of significance.

Results

1.1. Root imaging and plant biomass

In **Table 2**, outputs from the two-way ANOVA for root growth and plant biomass of wheat seedlings of the two investigated genotypes submitted to bacterial inoculation are reported.

Table 2. Statistical significance of F values from two-way analysis of variance for total, lateral and primary root length, total root surface area, root and shoot biomass.

Source of Variance	DF	Total root length (cm)	Total lateral root length (cm)	Total primary root length (cm)	Total root surface area (cm^2)	Root biomass (mg DW)	Shoot biomass (mg DW)
Inoculum	2	***	***	***	***	***	***
Genotype	1	ns	ns	ns	ns	ns	ns
Inoculum x Genotype	2	*	*	*	ns	ns	ns

DF: degrees of freedom; *, **, ***: statistically significant at P≤0.05, P≤0.01 and P≤0.001 probability level, respectively; n.s: not significant.

A highly significant effect of inoculum but no effect of genotype was observed for all examined variables. Furthermore, the interaction inoculum x genotype was significant only for root length (**Table 2**).

Table 3. Effect of different bacterial inoculation (*E. coli DH5α*, 12A-*Bacillus* sp., 25A-*Bacillus* sp.) on root growth and surface of wheat seedlings (*cv* Simeto and Cadenza) grown in low P soil.

		Total root length (cm)	Total lateral root length (cm)	Total primary root length (cm)	Total root surface area (cm^2)
Inoculum	E. coli DH5α	529.146^{B}	377.468^{A}	144.261^{B}	50.291^{B}
	12A	784.80^{A}	506.419^{A}	251.153^{A}	72.15^{A}
	25A	541.60^{B}	379.155^{B}	159.621^{B}	54.68^{B}

Data are reported as mean values (n=8). Different letters indicate significant differences at P≤0.001 according to Tukey's test. ns: not significant

Compared to no-PGPB (*E. coli DH5α*) inoculated plants, the strain 12A promoted a highly significant increase of root parameters while the inoculation of plants with the 25A strain had no effect. In particular, the total root length of the 12A- inoculated plants was 48.3% longer than the no-PGPB control plants. A significant increase was also observed for lateral and primary root length (+34.2% and 74% respectively). Therefore, the total root area was significantly greater (+50.7%) than that of control plants (**Table 3)**.

B)

A)

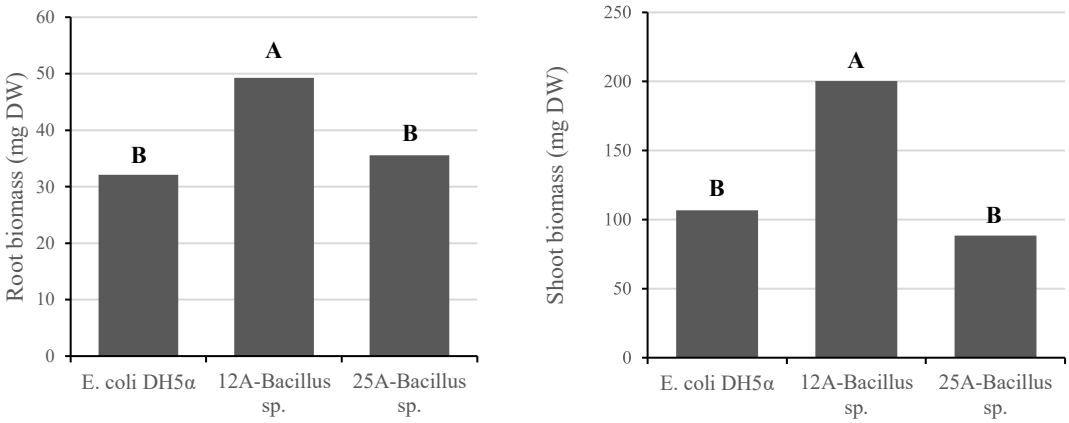

Figure 1. Effect of bacterial inoculum (*E. coli DH5α*, 12A-*Bacillus* sp., 25A-*Bacillus* sp.) on wheat seedlings biomass grown in low P soil. A) Root dry weight; B) shoot dry weight. Data are reported as mean values (n=8). Different letters indicate significant differences at P≤0.001 according to Tukey's test.

Consistent with the root scan results, the averages of root and shoot dry weight (DW) of the plants inoculated with the 12A strain were significantly (P≤0.001) greater than in plants inoculated with *E. coli DH5α* (+ 53.4% and + 87.8% respectively) (**Figure 1A, B**).

Furthermore, the treatment with the 12A strain produced the maximum shoot P content (0.86 mg/g DW), which was significantly (P≤0.05) higher than obtained with the 25A *and E. coli DH5α* treatments (0.66 mg/g DW and 0.63 mg/g DW respectively).

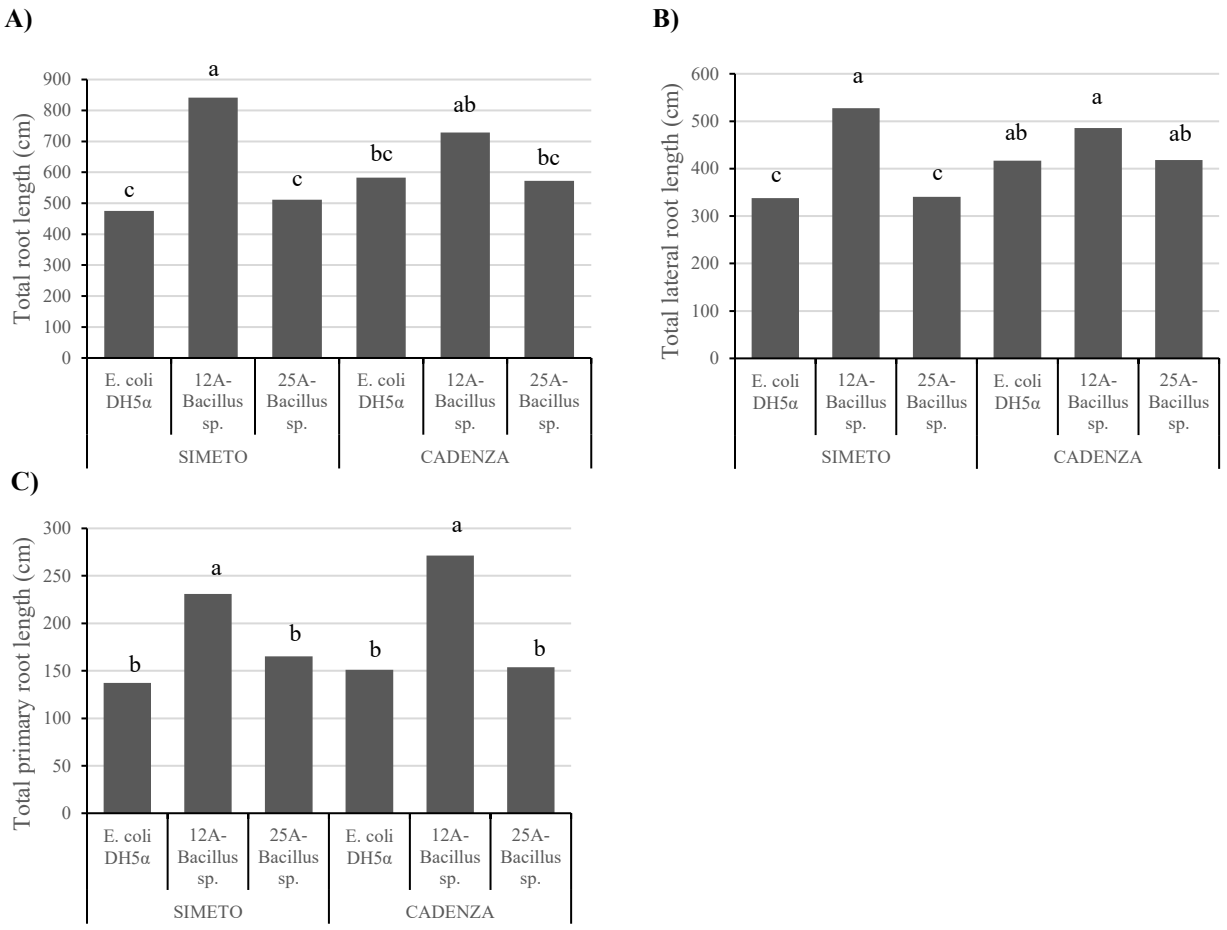

Figure 2. Effect of the interaction of bacterial inoculum (*E. coli DH5α*, 12A-*Bacillus* sp., 25A-*Bacillus* sp.) with genotype (*cv* Simeto and Cadenza) on root growth of wheat seedlings grown in low P soil. Data are reported as mean values (n=4). Different letters indicate significant differences at P≤0.05 according to Tukey's test.

Increased root growth and root surface area was evident in 12A- inoculated plants compared with *E. coli DH5α*- inoculated plants for both genotypes but significant only for root length (**Figure 2A, B, C**).

1.2. Reverse transcriptase (RT)-PCR

(RT)-PCR was used to evaluate a possible effect of PGPB inoculum on the expression of the genes IPS1 and *Ta*PT6 as indicators for Pi uptake and P nutrition under P starvation growth condition.

In **Figure 3,** the gene expression analyses in leaf samples are reported. The IPS1 gene was expressed in *E. coli DH5α*- inoculated plants, while it was not expressed in PGPB- inoculated plants (12A, 25A strains). *Ta*PT6 was induced in both control and PGPB- inoculated plants (**Figure 3A, B**).

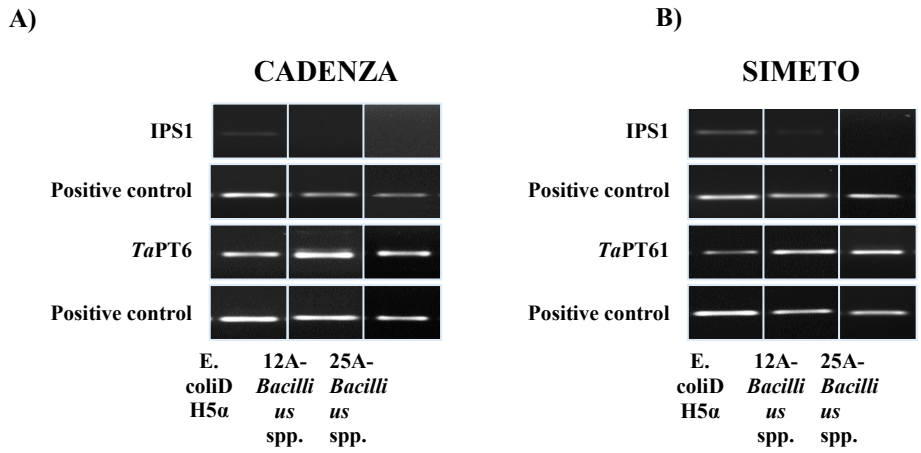

Figure 3. Expression pattern of IPS1, *Ta*PT6 in leaves of wheat seedlings grown in low P soil. A) Cadenza, B) Simeto. All semi-quantitative RT-PCR figures are representative of three biological replicates.

Discussion

In this study, the capacity of autochthonous PGPB inoculum to promote the growth of wheat seedlings in low P soil was evaluated. Among the two tested strains, only 12A significantly increased root length and surface and root biomass. The different behaviour of the two isolates seems consistent with their previous phenotypic characterization, evaluated *invitro*, since the 12A strain was able to produce IAA in addition to the P mineralizing and P solubilizing capability (Di Benedetto *et al*. 2019). The IAA production represents an important selection criteria for bacteria with regards to their application as bio-fertilizer for cultivated plants (Glick 2012; De Souza *et al*. 2015; Di Benedetto *et al*. 2017). Bacteria that produce IAA and promote plant growth were described for different crops such as maize, wheat, soybean and rice (Spaepen *et al*. 2008; Ali *et al*. 2009; Majeed *et al*. 2015; Di Benedetto *et al*. 2017). It is known that high levels of bacterial IAA are associated with lateral and adventitious root formation (Patten *et al*. 2002; Spaepen *et* al. 2008; Ribeiro *et al*. 2018). In our experiment, an increase in both the primary and lateral root length was reported which might be due to IAA production.

In accordance with an extended root surface, length and biomass, root colonization with the 12A strain (IAA producer) led to a better shoot growth (dry weight).That may be a consequence of an IAA response related to increased root branching and more efficient nutrient uptake by the plant. Also, some authors suggest a direct effect of IAA-producing bacteria on shoot growth (Dodd *et al*. 2010; Pérez-de-Luque *et al*. 2017; Ribeiro *et al*. 2018).

The positive effect of 12A inoculation on root and shoot dry weight of plants might result from the combination of increased root length and surface area and the P mineralizing and solubilizing ability as confirmed by a high P content.

On the contrary, under the experimental condition applied in this study, the 25A inoculum had no effect on root development despite its ability to mineralize and solubilize P. This might be explained, at least partially, by the bacterial inability to produce IAA. In addition, it is possible that the effect of the 25A strain was not sufficient to increase plant growth and the parameters investigated in our experiment because of a lower ability to mineralize P than 12A strain.

The second aim of this study was focused on the effect of the PGPB inoculation on the expression of two genes involved in phosphate nutrition in response to P deficiency growth condition. The IPS1 gene is highly responsive to P deficiency and its transcript level is a good marker for P deficiency responses and has been shown to be involved in P homeostasis (Shin *et al*. 2006; Franco-Zorrilla *et al*. 2007; Doerner 2008; Hammond and White, 2008; Liu *et al*. 2010). IPS1 transcripts prevent the formation of the miRNA399 silencing complex with PHO2 mRNA (an ubiquitin E2 conjugase) adjusting the transcript levels of PHO2, which is a key player in balancing Pi with respect to its supply and demand since it is responsible of P transporter degradation (Franco-Zorrilla *et al*. 2007; Briat *et al*. 2015; Kumar *et al*. 2017). The loss of function of IPS1 causes high P accumulation in shoots; inversely, the overexpression of IPS1 decreases P accumulation in shoots (Shin *et al*. 2006; Franco-Zorrilla *et al*. 2007), indicating that IPS1 is involved in Pi remobilization and translocation pathways. Our results showed expression of IPS1 in no PGPB- inoculated plants but not in the 12A and 25A inoculated plants. This suggests that activation of the Pi-mediated signalling cascade IPS1-miR399-UBC24/PHO2 in response to the severe Pi starvation growth conditions occurred in the *E. coli* control plants consistent with the reduction of Pi levels in the shoots of these control plants.

On the contrary, the absence of IPS1 transcripts in the 12A and 25A inoculated plants indicate less severe Pi-starvation of the plants. In 12A- inoculated plants a higher amount of Pi in the shoots was found (0.86 mg/g DW), suggesting a higher P-removing in pots that received 12A inoculants due to increased P availability in the rhizosphere through bacterial mineralization of organic P. Unlike 12A-inoculated plants, plants inoculated with the 25A strain (no IAA producer) did not show an increase in shoot P content despite the absence of IPS1 gene (0.66 mg/g DW). This might be due to both, a lower mineralizing capacity and a lower root branching due to its inability to produce IAA.

Furthermore, in accordance with the P-limiting growth condition, RT-PCR showed that the PT6 expression was induced in both control and PGPB- inoculated plants. This might suggest a higher P-uptake via PT6 transporters (**Figure 3**). These results are consistent with the fact the PT6 expression is induced by Pi limitation not only in roots but also in leaves, playing a dual role in Pi uptake from the soil and Pi translocation inside the plant (Ai *et al*. 2009; Lopez-Arredondo *et al*. 2014; Zhang *et al*. 2014).

Conclusion

The effect of soil inoculation with two autochthonous strains on wheat seedlings grown under P limiting conditions in a pot experiment was investigated. Between the two investigated strains, only 12A- *Bacillus* sp. significantly increased root length, root surface area and root biomass. Furthermore, an enhanced shoot dry weight and shoot P content was found which might be due to a combined positive effect on root length and surface area, as well as mineralization and solubilization of P. On the contrary, the 25A- *Bacillus* sp. strain did not stimulate root growth or increase plant biomass and P content in shoots, may be due to its inability to produce IAA and to a lower capacity to mobilize unavailable Pcompared with 12A- *Bacillus* sp. strain.

A longer-term experiment will be necessary to confirm the beneficial effect of soil inoculation with the 12A strain on P accumulation and shoot biomass. Furthermore, a deeper investigation on the expression of other key genes involved in the P-starvation response and Pi uptake will be necessary.

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6. General discussion and conclusion

Due to the increase in human population growth, the depletion of natural resources and the environment pollution, it is necessary to raise agricultural productivity without enhancing environmental footprint. Achieving yield increases reducing negative impacts on the environment is not easy to obtain. A sustainable intensification approach on arable lands may offer the prospect of increasing farm input efficiency, reducing fertilizer applications and so reducing the agricultural GHGs emissions. The issue of sustainability is even more acute in semi-arid and arid regions, such as Mediterranean arable lands, where drought and related biophysical factors create a fragile and unstable environment for production (Ryan *et al*. 2008). In Mediterranean area, durum wheat is the most extensively cultivated cereal, provides food security to a large population share and is mostly used for the production of pasta, couscous and bulgur (Royo *et al*. 2017). The first European producer of durum wheat is Italy (Vita *et al*. 2016) with a global production of 4.2 million tons and a surface of 1.27 million hectares. Most of durum wheat production comes from Southern Italy where Apulia Region has the largest cultivated surface in Italy (ISTAT 2018_http://agri.istat.it).

In the last few decades, the use of PGPB as bio-fertilizers has gained importance in order to reduce the environmental pollution, the agricultural GHGs emission and to preserve the P deficiency of soil and the limited availability and non-renewable nature of rock phosphate. Different studies demonstrate their beneficial role in the growth and yields of crop species like maize, rice and wheat, which represent the three first crops in terms of production in the world (FAO 2015). Despite in literature several studies are available on the use of commercial PGPB, the effect of autochthonous PGPB isolated from soils has been less investigated (**see Chapter 2**).The first goal pursued in this thesis was to review literature data on the application of commercial and autochthonous PGPB used as bio-fertilizers in cereals focusing on the interaction PGPB-wheat. We found that *Pseudomonas*, *Burkholderia*, *Azospirillum* and *Herbaspirillum* are the most frequently PGPB used as bio-fertilizers for these crops. In most cases their use as commercial formulations significantly increases crop parameters like grain yield, root and shoot length or plant weight in pot or field experiments (Riggs *et al*. 2001; Baset and Shamsuddin 2009; Rosas *et al*. 2009; de Salamone *et al*. 2012; Krey *et al*. 2013; Kumar *et al*. 2014; Dal Cortivo *et al*. 2017).A critical issue on the use of allochthonous formulates is that they could not possess some mechanisms of adaptive capacity to win the stressful environmental conditions at different ecological environments (Corbo*et al*. 2017). On the contrary autochthonous strains naturally possess some mechanisms of adaptive evolution. The use of native strains as inoculants, characterized and

selected for growth-promoting traits of interest, could represent an important tool to search for region-specific microbial strains which can be used as bio-fertilizers (Majeed*et al*. 2015). Thus, deeper investigation on the isolation and characterization of autochthonous strains is needed. To date the benefits of autochthonous PGPB in maize, rice and common wheat (Montañez *et al*. 2012; Zhao *et al*. 2014; Manzoor *et al*. 2017; Islam *et al*. 2009; Araújo *et al*. 2013 Park *et al*. 2007; Beneduzi *et al*. 2008; Nathan *et al*. 2011; Dewangan *et al*. 2014; Anitha *et al*. 2014; Shaharoona *et al*. 2008; Majeed *et al*. 2015; Rana *et al*. 2012) has been extensively investigated while at our best knowledge only few reports are available on the isolation, characterization and selection of PGPB from durum wheat and on their effect on plant growth and nutrient efficiency. In this thesis, a contribution to increase the knowledge on this item was given by:

i) thephenotypic and genotypic characterization of autochthonous non-pathogenic PGPB from durum wheat rhizosphere;

ii) the selection of two nitrifying strains with the potential to improve N use efficiency in durum wheat;

iii) the selection of onestrain with P-solubilizing and P-mineralizing combined capability with the potential to improve wheat plant growth submitted to P starvation.

***In vitro* characterization of autochthonous PGPB from durum wheat rhizosphere**

Four-hundred seventy-four bacteria isolated from the rhizosphere of durum wheat, were characterized *in vitro* (**see Chapter 3**). The *in vitro* characterization of several hundreds of strains was a critical phase to select isolates with desired growth-promoting traits and able to survive and establish in wheat rhizosphere to be used as bio-fertilizers. Criticisms concern: i) the choice of the most effective qualitative and quantitative methodologies to select PGPB; ii) the statistics adopted for the selection and iii) the validation method in soil of selected bacteria. Furthermore, the management of a high number of isolates could lead to a bias or an under-estimation of the sources of microbial diversity.

In literature, there is not an "isolation and characterization protocol" for selecting PGPB as bio-fertilizers. Thus, a “more rapid” combined method of *in vitro* tests, advanced statistical analysis and *in vivo* validation are missing. The main challenge of this PhD thesis was to reduce the complexity of the dataset but in the same time avoiding a significant loss of information. A second challenge was the definition of inclusion/exclusion criteria to reduce the number of the samples and to select the microorganisms with growth promoting traits of interest. A possible drawback in this step was the definition of too restrictive

inclusion criteria, thus excluding interesting microorganisms, or to define decision criteria, which are not able to reduce the complexity of the contingency table. Based on these challenges, we used an innovative selection protocol based on different steps, i.e. grouping the strains in functional classes, studying the diversity/heterogeneity inside each group, and performing a selection by using the most important variables like ammonification and nitrification capacity, P- solubilisation and P-mineralization processes as well as IAA production. In accordance with literature (Kumar *et al*. 2014; Singh and Lal 2016) we found that pseudomonads were the group with the highest NH_4^+ and NO_2^-/NO_3^- production capacity. Moreover, we observed for *Bacillus* spp. that 75% and 60% of the bacilli isolated were respectively NH_4^+ and NO_2^-/NO_3^- producers. The 65% of the isolates positive to phosphate solubilization belonged to pseudomonads. Also, we found that P solubilization and mineralization could coexist in the same bacterial strain in accordance with literature (Tao *et al*. 2008).Relatively to the regulator of plant growth we observed that 50% and 40% respectively of *Bacillus* spp and pseudomonads were IAA producers.
As a result of the "step by step" characterization, sixteen strains characterized by growth promoting traits of interest were selected and identified by 16S sequencing; they belonged to *Bacillus*, *Pseudomonas* and *Stenotrophomonas* consistent with the genera common isolated in soil (Rana *et al*. 2012; Sarker *et al*. 2014; Majeed *et al*. 2015). Eight isolates (12A, 25A, 36M, 40M, 97M, 6P, 20P and 23P) were selected and tested as inoculants in wheat seedlings for a preliminary validation in soil. Finally, three strains showed promising results in terms of growth enhancement (25A- *Bacillus* sp, 6P- *Stenotrophomonas* spp, 20P- *Stenotrophomonas* spp). Among them 6P and 20P strains were selected to evaluate, in a pot experiment, their effect on improving nitrogen use efficiency in durum wheat due to their NO_2^-/NO_3^- and NH_4^+ production capacity (**see Chapter 4**). 25A was selected due to the highest P-solubilizing and P-mineralizing combined capacity (**see Chapter 5**).

Effect of PGPB inoculation on nitrogen use efficiency in durum wheat

In literature, the positive effect of autochthonous PGPB is well documented in common wheat (Rana *et al*. 2012; Majeed *et al*. 2015), while, at our best knowledge durum wheat has been less investigated. In this study, a contribution to increasing knowledge on this item was given by investigating the effect of 6P and 20P in relation to nitrogen use efficiency in durum wheat. Consistently with Majeed *et al*. 2015, we observed that both *Stenotrophomonas* strains 6P and 20P showed a high capacity to colonize and survive in durum wheat rhizosphere, up to maturity stage showing the highest viable count at booting stage.Furthermore,at this phase, soil 6P and 20P counts were a logarithm cycle higher than in

control pots, which demonstrates the further ability to win and overcome the other bacteria present in soil.

In relation to N use efficiency, the effects of PGPB inoculation changed depending on genotype. In Saragolla both strains improved UPE and NUPE while, in Simeto only 20P strain showed a positive effect. The positive effect of 6P and 20P inoculum on N use efficiency suggests their potential use under low N input such as in our experimental conditions. Similar results were reported by Shaharoona *et al*. (2008) on the effect of *Pseudomonas* strains on N use efficiency in wheat (*Triticumaestivum*) under different levels of N fertilizer (30, 60, 90 and 120 kg ha^{-1}), both in pot and field experiments. The authors reported that N use efficiency significantly increased in response to inoculation at all fertilizer levels but the maximum NUE value was observed under the lowest N level supplied.

The different behavior of 6P strain between genotypes highlighted the importance of plant-PGPB interaction as a key factor for the success and efficiency of PGPB inoculation. Also Iniguez *et al*.(2004) evaluating the effect of *Klebsiellapneumonia* strain 342 on N accumulation in three different wheat genotypes (*Triticumaestivum*), observed an increase in N content only in one genotype, while the others showed N deficiency symptoms.

Similarly, Neiverth *et al*. (2014) testing the effect of *Herbaspirillum* sp inoculation on five wheat (*Triticum aestivum*) genotypes, found that two genotypes showed increased shoot and/or root weight; three genotypes were either unaffected by inoculation or responded negatively with a decrease in shoot and root weight upon inoculation.

Effect of PSB and PMB isolates on plant growth of durum and bread wheat seedlings submitted to phosphate starvation.

The inoculation of soil with PSB and PMB could represent a promising strategy for the improvement of plant growth under P starvation (Singh and Kapoor 1999; Krey *et al*. 2013). Certain PSB and PMB may improve plant growth by increasing root surface area due to their ability to synthesize and export IAA. Despite some studies are available on the effect of PSB and PMB in relation to root growth promotion and the expression of key Pi- transporters (Kumar *et al*. 2014; Saia *et al*. 2015; Ogut *et al*. 2016), very little is known about the expression of key genes induced by P-starvation in PGPB-inoculated plants such as IPS1. IPS1 gene is highly responsive to P deficiency and its transcript determines high P accumulation in shoots; inversely, the overexpression of IPS1 decreases P accumulation in shoots (Shin *et al*. 2006; Franco-Zorrilla *et al*. 2007), indicating that IPS1 gene is

involved in Pi remobilization and translocation. To investigate the capacity of our best P- solubilizing and P- mineralizing isolates, 25A and 12A, to promote the growth of wheat seedlings in low P soil, a pot experiment was carried out. Interesting, 12A and 25A differed for the level of P- mineralization and for the production of IAA since the 12A strain was characterized by a higher P- mineralizing activity and by the IAA-production capacity. The expression of IPS1 in no PGPB- inoculated plants indicated a severe Pi starvation condition that resulted in the reduction of Pi levels in control shoots. On the contrary, the absence of IPS1 transcript might suggest a less Pi-starvation condition in plants inoculated with PGPB. Moreover, a different effect of PGPB strains was observed. In 12A- inoculated plants a higher amount of Pi in the shoots was observed suggesting a higher P-removing in pots that received 12A inoculants due to an increasing P availability in rhizosphere through bacterial mineralization of organic P. On the contrary, 25A inoculated plants did not show an increase in shoot P content despite the absence of IPS1 gene. It could be due to both a lower mineralizing capacity and to a lower root branching since its inability to produce IAA.

Conclusion

The use of PGPB is a frontier goal in soil science and microbiology even if the selection of a PGPB microorganism with specific desired properties is a complex process. In this thesis promising microorganisms were selected to be used as PGPB, for the improvement of N and P use efficiency in durum wheat. The research was performed in three phases: i) isolation and *in vitro* phenotypic characterization of PGPB from durum wheat rhizosphere with high potential to enhance nutrient use efficiency; ii) selection and genotypic identification of PGPB with the best performances; iii) validation on durum wheat seedlings grown in soil under controlled conditions.

Among the selected strains, 6P and 20P were identified because of their potential to improve NUEP in durum wheat. Also, one strain (12A) with P-solubilizing and P-mineralizing combined capability was identified for its potential use in improving wheat plant growth under P starvation.

Further experiments under field conditions and under different environments will be necessary to validate the effect of the selected strains on wheat nutrient use efficiency. Also a deeper investigation on the expression of key genes involved in N and Pi uptake will be necessary.

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