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**Corso di Dottorato in
Gestione dell'Innovazione nei Sistemi Agro-Alimentari della
Regione Mediterranea
XXXVII ciclo**

**Characterization of the insecticidal
activity of plant by-products of *Punica
granatum* (L.) and *Cynara scolymus* (L.)
against *Sitophilus granarius* (L.)
(Coleoptera, Curculionidae) and *Ephestia
kuehniella* (Zeller) (Lepidoptera,
Pyralidae)**

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*Alle radici che mi
hanno fatta crescere
e ai rami che sto per far germogliare.*

*A tutta la mia famiglia,
quella di sempre e quella che verrà.*

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Riassunto

Lo sviluppo di strategie di controllo innovative ed ecosostenibili nei confronti degli artropodi dannosi rappresenta oggi una priorità del settore agroalimentare, in linea con la Direttiva 2009/128/CE sull'uso sostenibile dei prodotti fitosanitari e con gli obiettivi del Green Deal europeo, che prevedono una riduzione del 50% dell'impiego dei pesticidi entro il 2030. Parallelamente, si pone la necessità di affrontare un'altra sfida globale del comparto agroalimentare, ovvero la gestione delle ingenti quantità di scarti e sottoprodotti generati lungo la filiera di trasformazione di frutta e ortaggi. Molti di questi sottoprodotti, tra cui bucce, semi, brattee e residui della lavorazione industriale, pur essendo generalmente destinati allo smaltimento, contengono elevate quantità di composti bioattivi con potenziali applicazioni nella protezione delle colture e delle derrate. Il recupero di tali matrici si inserisce pienamente negli obiettivi di sviluppo sostenibile delle Nazioni Unite in quanto consente di coniugare la riduzione degli sprechi con la disponibilità di composti naturali a basso impatto sulla salute umana e sull'ambiente.

Nell'ambito della protezione delle derrate, il punteruolo del grano *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) e la tignola grigia della farina *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) rappresentano due delle specie più dannose, rispettivamente come infestante primario dei cereali in granella e come infestante secondario delle farine e dei prodotti della macinazione e trasformazione. Il loro controllo si basa ancora prevalentemente sull'impiego di insetticidi di sintesi e fumiganti. La disponibilità di tali strumenti si è tuttavia progressivamente ridotta negli ultimi decenni, a causa delle restrizioni normative, della comparsa di fenomeni di resistenza e delle crescenti preoccupazioni legate ai residui chimici negli alimenti e alla tossicità per operatori e organismi non target. In questo contesto, vi è un interesse crescente verso l'individuazione di mezzi di controllo alternativi, efficaci e a basso impatto ambientale, tra cui l'impiego di composti di origine naturale.

L'obiettivo principale della tesi di dottorato è stato quello di valutare l'attività biologica di estratti ottenuti da due sottoprodotti della filiera agroalimentare mediterranea: le bucce di melograno (*Punica granatum* L.), scartate in grandi quantità dall'industria del succo e del concentrato, e le brattee esterne del carciofo (*Cynara scolymus* L.), eliminate nelle prime fasi della lavorazione conserviera e del surgelato.

Entrambe le matrici presentano un'elevata ricchezza in composti fenolici e altri metaboliti secondari, ma il loro potenziale nei confronti degli insetti infestanti delle derrate è stato finora indagato solo in misura limitata. L'attività sperimentale ha previsto inizialmente la preparazione di estratti grezzi con solventi di diversa polarità (acetone, *n*-esano ed etere dietilico), saggiati nei confronti degli adulti di *S. granarius* e delle larve di *E. kuehniella* in prove di tossicità per contatto e ingestione, saggi di repellenza e valutazione degli indici nutrizionali.

I risultati hanno evidenziato differenze marcate tra i due sottoprodotti. Le bucce di melograno, e in particolare l'estratto acetonic, hanno mostrato un'attività biologica ampia e articolata, comprendente tossicità per contatto e ingestione, effetti fagoderrente, interferenza con gli indici nutrizionali e repellenza su entrambe le specie target. Le brattee di carciofo hanno invece espresso un'attività più circoscritta, limitata prevalentemente alla tossicità per contatto.

L'individuazione dei composti responsabili dell'attività biologica dell'estratto acetonic delle bucce di melograno è stata condotta mediante un frazionamento bio-guidato, che ha combinato separazioni cromatografiche progressive e prove biologiche sulle frazioni ottenute. Il processo ha portato all'isolamento di un'unica frazione attiva nei confronti di entrambe le specie, successivamente purificata mediante cromatografia su strato sottile (TLC) fino a ottenere una subfrazione a composizione chimica semplificata. L'analisi gascromatografia accoppiata a spettrometria (GC-MS) ha evidenziato come componente principale uno sterolo appartenente alla classe dei sitosteroli. Il confronto con l'unico standard disponibile in commercio (β -sitosterolo) ha tuttavia mostrato differenze sia nel comportamento cromatografico sia nel profilo biologico, suggerendo che il composto attivo corrisponde ad un isomero strutturale del β -sitosterolo.

Nell'ambito delle attività multidisciplinari svolte durante il dottorato, sono stati condotti ulteriori studi riconducibili ai diversi pilastri della difesa integrata (IPM), riguardanti: 1) l'influenza dei fattori ambientali sull'efficacia degli insetticidi; 2) l'impiego di polveri inerti come strumenti di controllo; 3) gli effetti dei composti volatili di origine vegetale sul comportamento degli insetti. In particolare, durante il periodo di ricerca all'estero è stata valutata l'influenza della temperatura, della disponibilità di cibo e della dose di applicazione sull'efficacia di λ -cicalotrina nei confronti di larve e adulti di *Alphitobius diaperinus* (Panzer), evidenziando come la suscettibilità dell'insetto dipenda dall'interazione tra fattori ambientali e biologici piuttosto che rappresentare una

proprietà intrinseca della specie.

Nel corso del dottorato sono state inoltre valutate l'efficacia e la persistenza di polveri inerti, tra cui una zeolite di origine cubana e una terra di diatomee, nei confronti di *S. granarius* su frumento. Tali studi sono stati integrati da un'indagine biochimica sui marcatori dello stress ossidativo e sul pathway della gliossalasi, al fine di chiarire il possibile meccanismo d'azione della zeolite.

Sono state, infine, analizzate le risposte comportamentali di *S. granarius* e *Rhyzopertha dominica* (F.) nei confronti dei composti organici volatili emessi da genotipi di frumento antichi e moderni, evidenziando differenze di attrattività tra le varietà, riconducibili a specifiche componenti dei profili volatili (VOCs). Studi successivi condotti su frumenti antichi (farro monococco e dicocco) hanno inoltre messo in evidenza differenze specie-specifiche nella suscettibilità e nelle preferenze d'ospite, indicando un ruolo rilevante delle caratteristiche varietali nella dinamica delle infestazioni.

Nel complesso, le attività svolte durante il dottorato hanno dimostrato che le bucce di melograno rappresentano una fonte concreta e promettente di composti naturali utilizzabili per il controllo degli insetti delle derrate, e che l'attività biologica osservata è riconducibile a uno specifico sterolo con caratteristiche distintive, la cui identificazione definitiva richiede ulteriori approfondimenti analitici. I risultati ottenuti sulle brattee di carciofo, pur evidenziandone un'efficacia più limitata, contribuiscono a delineare con maggiore realismo le potenzialità e i limiti dei diversi sottoprodotti vegetali nella protezione post-raccolta. Gli studi complementari ampliano il quadro degli approcci disponibili, confermando che lo sviluppo di strategie di controllo a basso impatto richiede l'integrazione di approcci diversi, tra cui estratti vegetali, polveri inerti, segnali chimici e gestione ambientale, in linea con i principi della lotta integrata e dell'economia circolare.

Abstract

The development of innovative and environmentally sustainable control strategies against harmful arthropods has become a major priority in the agri-food sector, in line with Directive 2009/128/EC on the sustainable use of plant protection products and the objectives of the European Green Deal, which aim to reduce pesticide use by 50% by 2030. At the same time, the agri-food industry has to address another major global

challenge, namely the management of the large quantities of waste and by-products generated along the fruit and vegetable processing chain. Many of these by-products, including peels, seeds, bracts and industrial processing residues, although typically discarded, often contain substantial amounts of bioactive compounds with potential applications in crop and stored-product protection. The valorisation of these residues is fully in line with the Sustainable Development Goals of the United Nations, as it combines waste reduction with the availability of natural compounds characterised by a low impact on human health and on the environment.

In stored-product protection, the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) and the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) are two of the most damaging species, acting respectively as a primary pest of whole-grain cereals and as a secondary pest of flours and milled products. Their control still relies largely on synthetic insecticides and fumigants, whose availability has progressively declined due to regulatory restrictions, the development of resistance, and growing concerns about chemical residues in food and about toxicity to operators and non-target organisms. This scenario has led to an increasing demand for alternative, effective, and low-impact control tools, including the use of plant-derived compounds.

The main objective of the PhD thesis was to evaluate the biological activity of extracts obtained from two by-products of the Mediterranean agri-food chain: pomegranate peels (*Punica granatum* L.), discarded in large amounts by the juice and concentrate industry, and the outer bracts of globe artichoke (*Cynara scolymus* L.), removed during the early stages of the canning and freezing process. Both matrices are characterised by a high content of phenolic compounds and other secondary metabolites, although their potential against stored-product insect pests has so far received only limited attention. The experimental study initially focused on the preparation of crude extracts with solvents of different polarity (acetone, *n*-hexane, and diethyl ether), which were tested against *S. granarius* adults and *E. kuehniella* larvae through contact and ingestion toxicity bioassays, repellency tests, and the evaluation of nutritional indices.

The two by-products showed markedly different biological profiles. Pomegranate peels, and in particular the acetone extract, showed a broad and articulated biological activity, including contact and ingestion toxicity, antifeedant effects, interference with nutritional

indices, and repellency against both target species. In contrast, artichoke outer bracts showed a narrower activity, mainly limited to contact toxicity.

The identification of the compounds responsible for the biological activity of the pomegranate acetone extract was carried out through a bioassay-guided fractionation, which combined sequential chromatographic separations with biological testing of the resulting fractions. This process led to the isolation of a single active fraction effective against both species, which was further purified by thin-layer chromatography (TLC) to obtain a subfraction with a simplified chemical composition. Gas chromatography-mass spectrometry (GC-MS) analysis identified a sterol of the sitosterol class as the main constituent. However, the comparison with the commercial standard revealed differences in both chromatographic behaviour and biological activity, suggesting that the active compound is not a β -sitosterol, but rather a structurally related isomer.

In parallel with the main research line, during the PhD, further studies were conducted in relation to different pillars of Integrated Pest Management (IPM), concerning: 1) the influence of environmental factors on insecticide efficacy; 2) the use of inert dusts as control tools; 3) the effects of plant-derived volatile compounds on insect behaviour.

In particular, during the mandatory research period abroad, the effects of temperature, food availability, and application dose on the efficacy of λ -cyhalothrin against larvae and adults of *Alphitobius diaperinus* (Panzer) were investigated, showing that insect susceptibility is shaped by the interaction between environmental and biological factors rather than representing an intrinsic species trait.

In addition, the efficacy and persistence of inert dusts, including diatomaceous earth and natural zeolites, were evaluated under different conditions, highlighting their potential as sustainable control tools, complemented by a biochemical investigation on oxidative stress markers and on the glyoxalase pathway as a possible mechanism of action of the zeolite.

Finally, the behavioural responses of *Sitophilus granarius* and *Rhyzopertha dominica* (F.) to volatile organic compounds (VOCs) emitted by old and modern wheat genotypes were analysed, revealing differences in varietal attractiveness. Complementary studies on ancient wheat varieties further showed species-specific differences in susceptibility and host preference, highlighting the role of wheat traits in influencing pest behaviour and infestation dynamics, with implications for sustainable pest management.

Overall, the activities carried out during the PhD have demonstrated that pomegranate

peels represent a promising source of natural compounds for the control of stored-product insect pests, and that the observed biological activity can be ascribed to a specific sterol with distinctive features, whose definitive identification will require further analytical work. The results obtained with artichoke outer bracts, although showing more limited efficacy, provide a clearer understanding of the potential and limitations of different plant by-products in stored-product protection. The complementary studies further broaden the range of available tools, confirming that the development of low-impact control strategies requires the integration of different approaches including plant extracts, inert dusts, chemical signals and environmental management, in line with the principles of integrated pest management and circular economy.

Thesis outline

Chapter 1 - Introduction

The introductory chapter focuses on the global relevance of stored-product insect pests for food security and the agri-food economy, with particular emphasis on the damage caused to cereal commodities and processed products. The biology, ecology, and infestation strategies of two of the most economically important stored-product pest species, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), are described in detail, including their life cycles, feeding behaviour, reproductive strategies, and impact on grain and flour quality.

Chapter 2 - Sustainable control means of stored-product insect pests

In this chapter, the urgent need for sustainable alternatives to synthetic insecticides within Integrated Pest Management (IPM) strategies is addressed. A comprehensive analysis of available low-impact control tools is presented, including physical methods, biological control agents, semiochemical-based approaches, and plant-derived insecticidal compounds. Particular attention is given to plant extracts and essential oils, including their modes of action, spectrum of biological activity, advantages, limitations, and potential integration into IPM programmes for stored-product protection.

Chapter 3 – Agro-Industrial By-Products, Bioactive Molecules, and Circular Economy

The third chapter explores agro-industrial by-products as sources of bioactive compounds for sustainable pest management, in line with circular economy and waste valorisation principles. Following an overview of the broader context of plant-derived residues in Mediterranean food processing systems, the chapter focuses on the phytochemical composition, ecological role, extraction potential, and documented or potential insecticidal, antifeedant, and repellent properties of pomegranate (*Punica granatum* L.) peel and globe artichoke (*Cynara cardunculus* var. *scolymus*) outer bracts, the two agro-industrial matrices investigated in the research articles included in this thesis.

Chapter 4 - Aim of the thesis

The objectives of the PhD thesis are described in a dedicated chapter. Briefly, the present thesis was mainly focused on characterising the insecticidal, antifeedant, and repellent properties of extracts from pomegranate peel and globe artichoke outer bracts against *S. granarius* and *E. kuehniella*, with the aim to identify the most bioactive solvent extracts, assess whether the two plant matrices differ in the nature and extent of their biological effects, and isolate the compounds responsible for the observed activity through a bioassay-guided fractionation approach. Additionally, studies were carried out to evaluate the influence of abiotic and biotic factors on the efficacy of λ -cyhalothrin against *Alphitobius diaperinus*, and to investigate other aspects of stored-product pest management including behavioural responses to cereal VOCs, and the insecticidal activity of inert dusts against different pest species.

Chapter 5 - Insecticidal and Repellent Activity of Pomegranate Peel Extracts Against *Sitophilus granarius*

This chapter is based on the published paper by Lo Muzio et al. (2025) in *Insects*, 16(12), 1222. The study investigates the bioactivity of pomegranate peel extracts obtained with solvents of different polarity (acetone, *n*-hexane, and diethyl ether) against adults of *S. granarius*, combining contact and ingestion toxicity assays with antifeedant, nutritional, and repellency evaluations. The results show clear differences among extracts, with the acetone extract consistently exhibiting the highest biological activity, while *n*-hexane displayed moderate effects and diethyl ether was largely ineffective. In particular, the acetone extract significantly affected insect survival, feeding behaviour, and food utilization. The GC-MS analysis revealed a phytochemical profile dominated by sterol derivatives and fatty acids, suggesting a potential role of these compounds in the observed activity. Overall, the study highlights the relevance of pomegranate peel as a promising source of bioactive compounds for the sustainable management of *S. granarius*.

Chapter 6 - Biological Activity of Pomegranate Peel for the green management of

Ephestia kuehniella (Lepidoptera: Pyralidae) larvae

This chapter is based on the published paper by Lo Muzio et al. (2026) in *Crop Protection*, 107729. The study investigates the insecticidal, antifeedant, nutritional, and repellent effects of pomegranate peel extracts obtained with solvents of different polarity against larvae of *E. kuehniella*. The results highlight the higher efficacy of the acetone extract compared to the other solvents, affecting larval survival, feeding behaviour, and development. The GC-MS analysis revealed a complex phytochemical profile, mainly composed of sterol derivatives, fatty acids, and phenolic compounds. Overall, the study supports the potential of pomegranate peel as a source of bioactive compounds for the integrated management of *E. kuehniella*.

Chapter 7 - Bioassay-Guided Fractionation and Identification of the Active Component of Pomegranate Peel Extracts

This chapter is based on a manuscript by Lo Muzio et al. (2026) submitted to *Scientific Reports*. Following the demonstrated bioactivity of the acetone extract of pomegranate peel, this study aimed to isolate and characterise the constituents responsible for its insecticidal effects against *S. granarius* adults and *E. kuehniella* larvae. A bioassay-guided fractionation approach was applied, combining silica gel column chromatography with sequential toxicity screening, leading to the identification of a single active fraction. Further purification by preparative TLC yielded one subfraction consistently toxic to both species. The GC-MS analysis showed that this subfraction was dominated by a sterol of the sitosterol family whose chromatographic behaviour did not match β -sitosterol, suggesting the presence of a structurally related isomer. Comparative bioassays with commercial β -sitosterol confirmed a markedly different biological profile. This study represents the final step of a bioassay-guided workflow aimed at elucidating the chemical basis of pomegranate peel activity and identifying candidate molecules for further investigation.

Chapter 8 - Evaluation of globe artichoke (*Cynara cardunculus* L.) outer bract extracts against *Sitophilus granarius* (L.)

This chapter is based on a manuscript by Lo Muzio et al. (2026) submitted to *Archives of Phytopathology and Plant Protection*. The insecticidal potential of globe artichoke outer bracts, a lignified agro-industrial by-product, was evaluated against adults of *S. granarius* through contact and ingestion bioassays. The acetone extract showed the highest contact toxicity, whilst the *n*-hexane extract was less effective and diethyl ether largely inactive. Ingestion assays did not reveal significant effects on mortality, feeding activity, or nutritional indices, indicating that the bioactive constituents of artichoke outer bracts act primarily through contact exposure and display a more limited biological profile compared with pomegranate peel. Overall, the study provides the first evidence that globe artichoke outer bracts represent a biologically active residue with potential for valorization in sustainable stored-product pest management.

Chapter 9 - Effect of Temperature, Food Availability and Dose on the Efficacy of λ -Cyhalothrin Against *Alphitobius diaperinus*

This chapter is based on the published paper by Lo Muzio et al. (2023) in *Insects*, 14, 1040. In this study, carried out during the mandatory research period abroad, the efficacy of λ -cyhalothrin was evaluated against larvae and adults of *A. diaperinus* under different temperature conditions (20, 25, 30, and 35 °C), food availability levels, and two application doses. Results demonstrated that insecticide efficacy is strongly influenced by abiotic and biotic factors: mortality increased significantly with temperature in both life stages, food availability markedly reduced exposure to insecticide residues and decreased mortality, and larvae were consistently more susceptible than adults. The study provides new insights into the environmental and biological factors that modulate the performance of synthetic insecticides against *A. diaperinus*, offering practical information for optimizing control strategies.

Chapter 10 - Insecticidal activity and persistence of a natural Cuban zeolite and a diatomaceous earth against *Sitophilus granarius* on wheat

This chapter is based on a manuscript by D'Isita et al. (2026) submitted for publication to *Journal of Plant Diseases and Protection*. The study compared the insecticidal

activity and persistence of a natural Cuban zeolite and a diatomaceous earth against adults of *S. granarius* on wheat. Mortality bioassays were conducted at increasing doses and progeny suppression was also assessed. Persistence tests were carried out up to 12 months after treatment. Both inert dusts were effective in inducing adult mortality and suppressing progeny, with diatomaceous earth generally acting faster and at slightly lower doses. Both materials maintained insecticidal activity over extended storage periods, confirming the practical potential of natural Cuban zeolite as a persistent low-impact tool for stored grain protection.

Chapter 11 - First insights into the insecticidal mechanism of natural zeolite against *Sitophilus granarius*: effects on the antioxidant defence system and glyoxalase pathway

This chapter is based on a manuscript by Laus et al. (2026) submitted to *Scientific Reports*. The study investigated, for the first time, whether the insecticidal activity of natural Cuban zeolite against *S. granarius* involves oxidative and dicarbonyl stress mechanisms. Adults exposed to different zeolite doses were analysed for markers of antioxidant status including reduced and oxidised glutathione, antioxidant enzyme activities, and glyoxalase I activity. Results showed that zeolite exposure altered the antioxidant balance and activated the glyoxalase pathway in exposed insects, supporting the hypothesis that oxidative stress contributes to the biological effects of this inert dust and extending its interpretation beyond simple cuticular desiccation.

Chapter 12 - Other studies on stored-product pest management

This chapter includes two complementary studies on the interactions between cereal genotypes and stored-product insect pests. The first study, published by D'Isita et al. (2024) in *Journal of Stored Products Research*, investigated the behavioural responses of *Sitophilus granarius* and *Rhyzopertha dominica* to volatile organic compounds emitted by old and modern wheat genotypes. The second study, published by D'Isita et al. (2026) in *Journal of Pest Science*, evaluated the susceptibility and attractiveness of ancient wheat varieties to the same pest species. Overall, these studies provide additional information on the role of cereal genotype traits in influencing pest behaviour, host preference and infestation risk, contributing to sustainable stored-product pest management strategies.

Chapter 13 - General discussion and conclusion

The main findings obtained in the various research activities of the PhD thesis are synthesised and discussed in a concluding chapter. Future perspectives arising from the acquired knowledge are also outlined.

1. Introduction

1.1 The stored-product insect pests

The storage of agricultural commodities has long been associated with insect infestation, as documented by archaeological evidence from early grain storage systems (Buckland, 1981; Panagiotakopulu, 2001). The establishment and persistence of stored-product insects across postharvest systems worldwide are primarily related to the availability of concentrated food resources, relatively stable microclimatic conditions, and reduced pressure from natural enemies (Linsley, 1944; Levinson & Levinson, 1994; Rees, 2004). In addition, their ecological success is supported by biological and ecological characteristics that enable efficient exploitation of storage environments (Subramanyam & Hagstrum, 1996; Phillips & Throne, 2010).

Many stored-product insect species develop efficiently at temperatures between 25 and 35°C, typical of tropical and subtropical storage conditions, and are able to exploit substrates with low moisture content as well as a wide range of commodities, including cereals, legumes, oilseeds, and processed products (Throne, 1994; Fleurat-Lessard, 2004; Stejskal et al., 2019). Population development is further favoured by relatively short generation times and extended adult longevity, allowing the formation of multiple overlapping cohorts within a single storage season. At the same time, small body size and the occurrence of cryptic developmental stages facilitate colonization during harvest, transport, and storage, often limiting the effectiveness of early detection and control measures (Hagstrum & Subramanyam, 2006; Hagstrum et al., 2012; Jian, 2019). Insect activity within stored commodities causes both quantitative and qualitative damage. Postharvest losses associated with insect infestation are estimated to range from 5-10% in industrialised systems to over 20-30% in developing regions, depending on storage conditions and pest management practices (Hodges et al., 2011; Suleiman & Rosentrater, 2022). Direct feeding reduces grain weight and structural integrity, while biochemical and structural degradation of kernels reduces milling efficiency, flour quality, and seed viability (Longstaff, 1981; Trematerra & Throne, 2012; Yaseen et al., 2019; Tadesse et al., 2020; Stathas et al., 2023). External feeders, including tenebrionid and cucujid species, preferentially attack broken kernels and grain dust, contributing to nutritional deterioration and increasing the proportion of unmarketable material (Stejskal et al., 2014). Insect metabolic activity increases grain temperature and

interstitial relative humidity within the grain bulk, promoting the proliferation of mycotoxigenic fungi, particularly *Aspergillus* and *Fusarium* species, and the consequent accumulation of mycotoxins of public health concern (Magan et al., 2003; Magan & Aldred, 2007; Pitt & Hocking, 2009). Physical contamination with frass, cast exuviae, silk threads, body fragments, and chemical secretions further compromise hygienic standards and reduces the commercial acceptability of stored products (Rajendran, 2020). The strict tolerance limits imposed by European Union regulations and international trade standards on contamination and mycotoxin levels frequently result in the rejection of non-compliant consignments and significant economic losses (Stejskal et al., 2014; Phillips & Throne, 2010).

Stored-product insects are commonly classified as primary or secondary according to their feeding behaviour and their ability to exploit intact or damaged substrates. Primary pests are able to infest whole, sound kernels and complete their development within the grain, whereas secondary pests depend on previously damaged material and contribute to the progressive deterioration of stored commodities (Guru et al., 2022; Demis & Yenewa, 2022). This distinction has important implications for monitoring and management, since primary pests often remain concealed within kernels during the early stages of infestation, complicating early detection and timely intervention (Hagstrum et al., 2012; Phillips & Throne, 2010).

Among Coleoptera, both larvae and adults contribute to grain damage, whereas in Lepidoptera feeding activity is restricted to the larval stage (Imura, 1990; Hagstrum et al., 2012). Among the most economically damaging primary pests are the grain weevils *Sitophilus granarius* (L.), *S. oryzae* (L.), and *S. zeamais* (Motsch.) (Curculionidae), whose larval stages develop entirely within cereal kernels (Longstaff, 1981; Hagstrum et al., 2012). Other primary pests of considerable economic importance include *Rhyzopertha dominica* (F.) and *Prostephanus truncatus* (Horn) (Bostrichidae), particularly in maize storage systems in tropical regions (Winterbottom, 1922; Campbell & Sinha, 1976; Hodges, 1986; Edde, 2012).

Species of the genus *Tribolium* (Tenebrionidae), including *T. castaneum* (Herbst) and *T. confusum* J. du Val, are among the most cosmopolitan and widespread secondary pests in stored grain systems globally, exhibiting a broad host range, high fecundity, and documented resistance to multiple insecticide classes, including phosphine and pyrethroids (Rees, 2004; Benhalima et al., 2004; Opit et al., 2012; Nayak et al., 2020).

In several major coleopteran pests, males emit aggregation pheromones, including sitophilure in *S. granarius* and *S. oryzae* (Faustini et al., 1982; Phillips et al., 1989) and dominicalure-1 and dominicalure-2 in *R. dominica* (Williams et al., 1981). These pheromones play a key role in mediating aggregation, mating, and oviposition site selection (Khorramshahi & Burkholder, 1981; Edde, 2012).

Within Lepidoptera, the families Pyralidae and Gelechiidae include the principal storage moths, such as *Plodia interpunctella* (Hübner), *Ephestia kuehniella* (Zeller), *Cadra cautella* (Walker), and *Sitotroga cerealella* (Olivier). These species produce silken webbing that binds grain particles together and reduces airflow within the grain mass, leading to localized moisture accumulation and favouring mould development (Rees, 2004; Shankar & Abrol, 2012). However, *Sitotroga cerealella* larvae complete their entire development within intact kernels and therefore act as a primary pest (Trematerra & Gentile, 2002; Hagstrum et al., 2012).

1.2 Biology and ecology of *Sitophilus granarius* (L.) and *Ephestia kuehniella* (Zeller)

The granary weevil, *Sitophilus granarius* (Coleoptera: Curculionidae), is considered one of the earliest associates of human grain storage and one of the most destructive primary pests of stored cereals. Archaeological findings indicate its presence in human settlements since the early stages of agriculture, with adult and larval remains recovered from Neolithic storage pits in Europe and the Near East (Panagiotakopulu & Buckland, 2018; Plarre, 2013). Because it cannot survive outside stored-grain environments, *S. granarius* is regarded as a fully synanthropic species whose worldwide distribution has largely resulted from centuries of grain trade and human-mediated dispersal (Levinson & Levinson, 1994; Panagiotakopulu & Buckland, 2017).

Adults exhibit a robust cylindrical body and a characteristic elongated rostrum typical of curculionid beetles. Body coloration ranges from reddish-brown to nearly black, and body length generally varies between 3.8 and 5.1 mm, including the rostrum. Adult size may vary depending on nutritional conditions and on the size of the grain kernel in which larval development occurs (Surtees, 1965; Longstaff, 1981). Unlike closely related species such as *S. oryzae* and *S. zeamais*, *S. granarius* is incapable of flight due to the fusion of the elytra and the reduction of the hindwings, a morphological condition

interpreted as an adaptation to the relatively stable conditions of grain storage environments (Longstaff, 1981; Rees, 2004).

The immature stages develop entirely within the grain kernel. Eggs are small, oval and whitish and are deposited singly inside the grain endosperm. After hatching, larvae are legless (apodous), creamy white and strongly curved, with a well-developed brown head capsule. Larval development proceeds through four instars, all of which feed internally on the grain endosperm until pupation occurs within the kernel. The pupal stage also takes place inside the grain, and adult emergence is marked by the appearance of characteristic round exit holes on the kernel surface. Because immature stages develop concealed within the kernel, infestations often remain undetected until adult emergence (Rees, 2004; Hagstrum et al., 2012).

Females perforate the kernel using their rostrum, deposit a single egg within the endosperm and seal the oviposition cavity with a gelatinous secretion that rapidly hardens. This plug may also function as an oviposition marker that reduces the likelihood of additional egg deposition in the same grain, thereby limiting larval competition (Levinson & Kanaujia, 1982).

The weevil feeds on a wide range of cereals, including wheat, barley, rye, oats, maize, millet and rice, as well as processed grain-based products (Hagstrum et al., 2012). Both larvae and adults consume the grain endosperm, causing quantitative losses in grain mass and qualitative deterioration that affect germination capacity and technological quality of cereals (Hagstrum et al., 2012; Athanassiou & Arthur, 2018). Internal feeding also predisposes kernels to invasion by secondary pests and microorganisms, accelerating the deterioration of stored grain (Dunkel, 1988).

Chemical communication plays an important role in the population dynamics of this species. Males release an aggregation pheromone, known as sitophilure, that attracts both sexes and promotes aggregation within grain masses, facilitating mate finding and the establishment of infestations (Phillips et al., 1989; Faustini et al., 1989).

The development and population growth of *S. granarius* are strongly influenced by environmental conditions, particularly temperature and grain moisture. Optimal development generally occurs at temperatures between 25 and 30 °C and grain moisture contents around 12–15% (Fields, 1992; Throne, 1994). Under these conditions the complete life cycle can be completed in approximately 30–45 days, allowing up to three or four generations per year in storage facilities (Fields, 1992; Throne, 1994; Rees,

2004). Adult longevity typically ranges from four to eight months depending on environmental conditions, allowing populations to persist within grain stores for extended periods (Rees, 2004; Hagstrum et al., 2012). In addition, *S. granarius* is capable of developing at relatively low temperatures compared with other stored-product insects, which contributes to its persistence in temperate storage environments (Fields, 1992).

Within the order Lepidoptera, the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is an important secondary pest of stored cereal products. Originally described by Zeller in 1879, it was historically referred to as *Anagasta kuehniella* in earlier literature before being reassigned to the genus *Ephestia* following taxonomic revisions (Rees, 2004). Unlike primary pests that attack intact kernels, this moth mainly infests processed commodities such as flour, semolina, bran and other milled cereal derivatives (Rees, 2004; Trematerra & Gentile, 2010). Its importance increased with the expansion of industrial flour milling, as large-scale processing facilities created favourable conditions for the establishment and spread of the species (Hill, 2002).

Adult moths have a wingspan of approximately 20–25 mm and are characterized by greyish forewings with darker transverse bands typical of pyralid moths (Rees, 2004). Although adults do not cause direct feeding damage, they play a key role in the spread of infestations within storage environments due to their ability to fly and colonize new substrates (Trematerra & Gentile, 2010).

Females lay their eggs directly on the surface of the food substrate, typically on flour or other finely milled cereal products. Eggs are oval, whitish and extremely small, measuring approximately 0.4 - 0.5 mm in length (Rees, 2004). A single female may lay several hundred eggs during its lifetime, allowing populations to increase rapidly under favourable environmental conditions (Simmons & Nelson, 1975). Egg development usually lasts about 3 - 5 days at temperatures around 25 - 30 °C (Mohandass et al., 2007).

Damage is caused exclusively by the larval stage. After hatching, larvae begin feeding immediately on fine cereal particles while producing silk threads that bind flour particles together. During feeding, larvae generate abundant silk webbing that aggregates flour into compact masses and contaminates the product with frass, exuviae and cast skins. These silk mats may also obstruct milling machinery and processing equipment, leading to substantial economic losses (Rees, 2004; Phillips & Throne, 2010). Larval

development usually includes five to seven instars depending on temperature, food quality and population density (Mohandass et al., 2007; Athanassiou & Arthur, 2018). Early instars are small and translucent, whereas later instars become progressively larger and more opaque, reaching approximately 12-15 mm in length before pupation (Rees, 2004). Under optimal environmental conditions larval development typically lasts between 20 and 30 days (Brindley, 1930; Rees, 2004). When larvae complete development they enter a characteristic wandering phase, during which they leave the feeding substrate and actively search for suitable pupation sites. During this stage larvae often migrate into cracks, crevices, packaging materials or structural elements of storage facilities, where pupation occurs within loosely constructed silk cocoons. The pupal stage generally lasts about 7-10 days under favourable conditions before adult emergence (Rees, 2004; Trematerra & Gentile, 2010). The adults are short-lived and do not feed (Norris, 1934).

The reproduction in *E. kuehniella* is mediated by a female-produced sex pheromone that attracts males and facilitates mate finding within storage environments. The principal pheromone component identified in this species is (Z,E)-9,12-tetradecadienyl acetate, which has been widely studied and is commonly used in monitoring systems for pyralid moths (Kuwahara et al., 1971; Corbet, 1973).

The life cycle of this pest is characterized by mean developmental times ranging from 42.8 to 59.8 days in soft wheat flours and from 40.3 to 42.1 days in durum wheat flours at 25 °C (Locatelli et al., 2008). In addition, the species is able to develop under relatively low moisture conditions typical of processed cereal products, which contributes to its persistence in dry industrial environments (Jacob et al., 1977).

Besides its economic importance as a pest, *E. kuehniella* is widely used as a model organism in applied entomology. Eggs of this species are commonly employed as a factitious host for the mass production of parasitoids, particularly species of the genus *Trichogramma*, which are widely used in biological control programs against lepidopteran pests (Moghaddassi et al., 2019).

2. Sustainable control means of stored-product insect pests

The management of stored-product insect pests has historically relied on a small number of synthetic fumigants and contact insecticides, with phosphine representing the backbone of chemical control in most grain storage and processing facilities worldwide (Scheff & Phillips, 2022; Nayak et al., 2020). The progressive phase-out of methyl bromide under the Montreal Protocol, due to its role in stratospheric ozone depletion, further consolidated this dependence by removing what had been an important alternative fumigant (Fields & White, 2002; Bell, 2014). Over time, however, the repeated and often intensive use of phosphine has led to the widespread selection of resistant populations in several major pest species. A recent global meta-analysis covering nearly 1,000 field populations documented that approximately 73% showed some degree of phosphine resistance, with *Rhyzopertha dominica*, *Sitophilus oryzae* and *Tribolium castaneum* among the most affected species worldwide (Silva et al., 2023). Resistance has also been reported in European storage contexts, including populations collected in Czech Republic grain stores (Aulicky et al., 2022), and its early detection is increasingly supported by molecular diagnostic tools (Sakka et al., 2024).

Alongside the problem of resistance, growing toxicological and regulatory concerns have contributed to a rethinking of how stored-product pests should be managed. The use of synthetic insecticides raises well-documented issues related to human health, ranging from acute occupational exposure to chronic dietary risks, as well as environmental contamination through residues in grain, dust and processing by-products (Sharma et al., 2019; Nayak et al., 2020). In the European Union, the list of active substances authorised for postharvest use has been progressively reduced and maximum residue limits in food have been tightened, reflecting both consumer demand for residue-free products and updated risk assessments (EFSA, 2023; Adler et al., 2022). Within the framework of integrated pest management along the “farm-to-fork” continuum, physical, biological, behavioural (semiochemical-based) and plant-derived control methods are combined to maintain pest populations below economic thresholds, representing three key pillars of non-chemicals alternatives or low-impact strategies in stored-product systems (Scheff & Phillips, 2022; Guru et al., 2022).

2.1 Physical control methods

Physical methods represent a heterogeneous group of control strategies that include temperature manipulation, modified atmospheres, structural exclusion and inert dusts (Fields, 1992; Navarro, 2012; Korunić, 1998; Scheff & Phillips, 2022). Their principal advantages lie in the absence of synthetic chemical residues on commodities and their general compatibility with organic production systems and strict food safety standards, which makes them particularly relevant in a regulatory context of progressively tightening maximum residue limits (Guru et al., 2022; Adler et al., 2022; Scheff & Phillips, 2022). However, their practical adoption is often constrained by the need for substantial infrastructural investments, specialised equipment and a high degree of operational discipline throughout the storage chain (Guru et al., 2022; Kavallieratos, 2025).

Temperature management

Temperature management represents a key physical strategy in stored-product pest control, as insect development is strongly influenced by thermal conditions. In this context, two complementary approaches are commonly adopted, based on the manipulation of temperature in opposite directions.

Grain cooling relies on the forced circulation of ambient air through the stored bulk by means of aeration systems, with the aim of reducing grain temperature to levels, generally below 15 °C, at which insect development is strongly suppressed, and population increase is effectively halted (Fields, 1992; Stejskal et al., 2019). Modern aeration systems are often equipped with temperature and relative humidity sensors connected to automated control units, which regulate fan operation and optimise cooling efficiency while preventing excessive moisture accumulation within the grain (Păun et al., 2021; Scheff & Phillips, 2022). This approach is primarily preventive and is most effective when integrated with proper sanitation measures and continuous monitoring; however, its applicability may be limited in warm climates, where ambient air does not allow sufficient cooling without the use of mechanical refrigeration (Guru et al., 2022). Conversely, heat treatment exploits the upper thermal limits of insects by exposing infested commodities or structures to temperatures typically ranging from 50 to 60 °C

for a sufficient period to ensure mortality across all developmental stages, including eggs and pupae, which are generally more tolerant (Fields, 1992; Porto et al., 2018). Structural heat treatments are widely adopted in food-processing facilities, such as flour mills, as an alternative to chemical fumigation, and usually require exposure times of 24-48 h to allow adequate heat penetration into concealed refuges, including cracks, crevices and machinery components (Dowdy, 1999; Tilley et al., 2007; Porto et al., 2018).

For stored commodities, dielectric heating technologies, including microwave and radio-frequency treatments, represent a promising alternative, as they enable selective heating of insects due to their higher water content compared with the surrounding grain matrix (Das et al., 2013; Taheri et al., 2022). Under controlled conditions, these methods have been shown to achieve high mortality levels without adversely affecting the nutritional quality of the product (Yadav et al., 2023; Bermudez-Aguirre & Niemira, 2023). Nevertheless, their large-scale application remains limited by high installation costs, difficulties in achieving uniform heat distribution, and the potential risk of thermal damage to sensitive commodities (Bermudez-Aguirre & Niemira, 2023; Taheri et al., 2022).

Modified and controlled atmospheres

Modified and controlled atmospheres (MAs/CAs) represent a well-established physical strategy for the disinfestation of stored commodities, based on the sensitivity of insects to altered gas compositions. Insect control is typically achieved by reducing oxygen concentrations to $\leq 2\text{-}3\%$, increasing carbon dioxide levels to $\geq 60\%$, or combining both conditions, depending on temperature, target species and developmental stage (Bailey & Banks, 1980; Navarro, 2012; Cao et al., 2019).

These atmospheres can be generated through different approaches, including the injection of industrial-grade CO_2 or N_2 , the use of oxygen scavengers, or passive mechanisms in hermetic storage systems (Ngoma et al., 2024). In hermetic conditions, the natural respiration of grain and associated microflora progressively reduces intergranular oxygen levels, leading to the establishment of hypoxia without external gas inputs (Navarro, 2012; Ngwenyama et al., 2022). This approach has gained increasing relevance with the development of gas-tight storage structures, such as

metallic silos, flexible polymer cocoons and triple-layer hermetic bags, which are applicable at both commercial and smallholder scale (Opoku et al., 2023; Ngwenyama et al., 2022).

The effectiveness of these systems is strongly influenced by storage conditions, particularly temperature and initial infestation levels. For example, temperatures around 30 °C have been shown to accelerate oxygen depletion and allow the establishment of lethal hypoxia within relatively short timeframes, often within ten days regardless of pest density (Donga & Baributsa, 2023). In addition, insect respiration itself contributes to oxygen consumption within sealed systems, as recently demonstrated for *S. granarius*, with implications for the optimisation of hermetic storage protocols (Müller-Blenkle & Adler, 2024).

Mortality primarily results from the disruption of aerobic metabolism under hypoxic conditions, combined with the toxic effects of elevated CO₂, and higher mortality is generally achieved when both factors are applied simultaneously (Cao et al., 2019; Cheng et al., 2013). Atmosphere-based approaches constitute an effective and sustainable alternative to chemical control, as they do not leave residues, are compatible with organic production systems, and can be applied over extended storage periods without compromising grain quality. However, their effectiveness depends on the maintenance of gas-tight conditions and requires continuous monitoring of gas composition (Navarro, 2012), while differences in susceptibility among species and developmental stages may necessitate prolonged exposure times, especially under low-temperature conditions (Cao et al., 2019; Cheng et al., 2013).

Structural measures, sanitation and packaging

Structural measures, sanitation and packaging constitute key components of physical pest management, primarily acting by preventing infestation and restricting pest access to stored commodities rather than directly inducing mortality (Morrison et al., 2019; Scheff & Phillips, 2022).

At the facility level, effective pest prevention relies on appropriate building design and maintenance. This includes the sealing of structural openings, the installation of insect-proof barriers and the implementation of air filtration systems to limit insect entry (Bell, 2014; Adler et al., 2022). These measures are complemented by rigorous

sanitation practices aimed at removing grain residues, dust and organic debris from equipment and storage environments where pest populations may persist; the interaction between sanitation and the efficacy of other IPM tactics has been extensively documented, demonstrating that poor hygiene conditions substantially reduce the effectiveness of fumigation, heat treatments and biological control by providing refugia that allow insect populations to survive treatment exposure (Bell, 2014; Scheff & Phillips, 2022; Morrison et al., 2019).

At the commodity level, packaging provides an additional barrier against re-infestation. High-barrier multilayer materials are widely used to protect processed products by limiting gas exchange and preventing insect penetration along the distribution chain (Stejskal et al., 2021). In addition, active packaging systems incorporating plant-derived repellent or insecticidal compounds can further enhance protection by enabling the controlled release of bioactive substances within the package headspace, thereby maintaining deterrent conditions without direct contact with the commodity (Pavela & Benelli, 2016; Sharma et al., 2022). Recent experimental evidence has confirmed that lipid-based formulations applied to different packaging substrates exert a measurable repellent effect against key stored-product beetle species, pointing to promising developments in this area (Shah et al., 2024).

Inert dusts

Inert dusts are mineral-based particulate materials widely used as physical insecticides in stored-product protection. This group includes diatomaceous earths, amorphous silica gels, zeolites and other clay minerals such as attapulgite, which differ in origin and physicochemical properties (Korunić, 1998; Subramanyam & Roesli, 2000; Zeni et al., 2021). The dust particles adsorb or abrade the epicuticular wax layer of the insect integument, disrupting the cuticular water barrier and inducing lethal desiccation through increased transepicuticular water loss (Korunić, 1998; Subramanyam & Roesli, 2000; Golob, 1997; Zeni et al., 2021).

The insecticidal efficacy of inert dusts is strongly influenced by a range of interacting factors. These include biological variables, such as insect species and developmental stage, and physicochemical characteristics of the dust, particularly particle size distribution, mineral composition and source (Korunić, 1998; Fields & Korunić, 2000;

Vayias & Athanassiou, 2004). Moreover, the environmental conditions, especially temperature and relative humidity, together with the duration of exposure, further modulate their effectiveness, with higher humidity generally reducing desiccation efficiency (Arthur & Puterka, 2002; Kavallieratos et al., 2005; Agraftoti et al., 2023). Notably, the effects of inert dust application are not limited to direct mortality: research has documented that treatment of grain with particulate materials can also disrupt the semiochemical interactions through which insects locate and assess host substrates, thereby reducing infestation pressure through a behavioural mechanism that is complementary to contact desiccation (Germinara et al., 2020).

Among inert dusts, diatomaceous earths are the most extensively studied and widely adopted materials for stored-product protection. They are characterised by low mammalian toxicity and are generally recognised as safe (GRAS) for use on food commodities, making them compatible with residue-free protection strategies. Their insecticidal efficacy, however, is influenced by both intrinsic properties and biological variability. In particular, inter-specific differences in susceptibility are well documented: species such as *Cryptolestes* spp. are highly sensitive, whereas *Sitophilus* spp. and *Oryzaephilus surinamensis* show intermediate responses, and *R. dominica* and *Tribolium* spp. are typically more tolerant, often requiring higher application rates to achieve effective control (Agraftoti et al., 2023).

Zeolites represent a structurally distinct but functionally related class of inert dusts. These hydrated aluminosilicates are characterised by a highly porous structure and, similarly to diatomaceous earths, exert their insecticidal activity primarily through adsorption of epicuticular waxes and subsequent desiccation, although their physicochemical properties differ substantially from those of diatom-derived materials (Eroglu et al., 2019; Agraftoti et al., 2023).

Their efficacy has been demonstrated against several stored-product insect species, including *S. oryzae* and *Oryzaephilus surinamensis*, where significant adult mortality and progeny suppression have been reported. However, insecticidal performance is generally more variable than that of diatomaceous earths and rarely reaches complete control, particularly under differing temperature and relative humidity conditions (Eroglu et al., 2019).

More recent studies have extended the evaluation of zeolite-based formulations to a broader range of taxa, including psocids, mites and anobiid beetles, highlighting their

potential role as complementary tools within integrated pest management programmes, especially in the management of secondary or less common infestations (Agrafioti et al., 2023).

Despite their broad-spectrum activity, inert dusts are generally slower acting than conventional insecticides, as mortality depends on progressive water loss rather than acute toxicity (Korunić, 1998; Zeni et al., 2021). For this reason, they are more appropriately applied as preventive tools or as components of integrated pest management strategies rather than as stand-alone curative treatments. Recent research has focused on improving their performance through combination with other control agents. In particular, the co-application of inert dusts with plant-derived compounds, such as essential oils, has been shown to enhance insecticidal efficacy and reduce application rates, likely through complementary mechanisms of action (Athanasidou et al., 2013; Campolo et al., 2014; Pavela & Benelli, 2016).

2.2 Biological control methods

Biological control in stored-product systems encompasses the deliberate use of predators, parasitoids, pathogens or antagonistic microorganisms to suppress pest populations (Iturralde-García et al., 2020; Guru et al., 2022; Güner et al., 2023). While historically less exploited than in field crops, biological control has gained renewed attention as part of broader efforts to reduce chemical inputs and enhance ecological sustainability in postharvest systems (Harush et al., 2021). The storage environment presents both opportunities and constraints for biological control. Its spatial confinement and relatively simple trophic structure may enhance host-natural enemy interactions; however, environmental conditions such as low relative humidity, limited refuges and frequent human interventions can reduce the effectiveness and persistence of natural enemies, particularly entomopathogens (Hervet & Morrison, 2021; Guru et al., 2022).

Parasitoids and predators

Several hymenopteran parasitoids have been studied as natural enemies of stored-product beetles and moths. Parasitoids such as *Anisopteromalus calandrae* (Howard), *Lariophagus distinguendus* (Förster) and *Theocolax elegans* (Westwood) attack immature

stages of *Sitophilus* spp., *Rhyzopertha* spp. and other internal feeders by ovipositing on or near the host concealed within the grain kernel (Adarkwah et al., 2014; Riudavets et al., 2023). For stored-product moths, egg and larval parasitoids such as *Trichogramma* spp. and *Habrobracon hebetor* (Say) have been evaluated for controlling *Ephestia kuehniella*, *Plodia interpunctella* in warehouses and packaged products (Nasir et al., 2017; Mbata & Warsi, 2019).

Predatory insects, such as certain Tenebrionidae and Carabidae, and predatory mites associated with storage ecosystems can also contribute to natural regulation of pest populations, although their use in deliberate augmentation programmes remains limited (Del Arco et al., 2025).

The main advantages of parasitoids and predators are their host specificity, potential for self-dispersal within storage structures, and compatibility with residue-free production systems. Major limitations include sensitivity to low relative humidity, interference from cleaning and structural treatments, and the need for careful coordination with any insecticide applications to avoid non-target impacts (Harush et al., 2021; Guru et al., 2022). Recent studies have highlighted that, in certain storage contexts, naturally occurring parasitoid communities can exert significant top-down control on pest populations, especially when synthetic pesticide use is reduced and structural conditions favour their establishment (Harush et al., 2021). This supports the idea of integrating conservation biological control into stored-product IPM.

Entomopathogenic microorganisms

Entomopathogenic fungi (EPF), bacteria, viruses and nematodes have been investigated as biological control agents against a range of stored-product insects. Among these, EPF such as *Beauveria bassiana* (Bals.-Criv.) Vuill. and *Metarhizium anisopliae* (Metchnikoff) Sorokin., have received particular attention due to their ability to infect insects via the cuticle, without requiring ingestion (Canhilal, 2016; Sharma et al., 2023). EPF spores can be applied as dry powders, oil formulations or wettable powders to grain or structural surfaces. Under suitable conditions of temperature and humidity, conidia germinate on the insect integument,

penetrate the cuticle, and proliferate in the haemocoel, eventually causing death. Sublethal infections may reduce feeding, fecundity and longevity, adding further regulatory effects (Irsad et al., 2023; Couceiro et al., 2025). However, the efficacy of EPF in stored products is often constrained by the low relative humidity typical of grain bulks and processing facilities. Formulation improvements, such as oil- based carriers and microencapsulation, as well as combination with other stressors (e.g. heat, diatomaceous earth), have been explored to enhance performance and consistency (Guru et al., 2022; Zeni et al., 2021). Entomopathogenic bacteria and viruses have seen more limited development in stored-product contexts compared to field crops, although *Bacillus thuringiensis* Berliner formulations have been tested against moth larvae in flour and packaged products, with variable success (McGaughey, 1978; Salama et al., 1991; Hamad et al., 2019). Overall, entomopathogens appear particularly promising for integration into multi-hurdle strategies rather than as stand-alone tools, especially in large commercial facilities where environmental conditions are tightly controlled (Rumbos & Athanassiou, 2017).

2.3 Semiochemical-based control methods

In insects, chemical communication is mediated by low-molecular-weight compounds that act as specific signals modulating behavioural and physiological responses. These compounds, collectively known as semiochemicals, include pheromones, which mediate intraspecific communication, and allelochemicals, which govern interactions between different species. Through these signals, key processes such as aggregation, mate finding, host selection and oviposition are finely regulated (Law & Regnier, 1971; Nordlund & Lewis, 1976; Dicke & Sabelis, 1988).

In stored-product systems, semiochemicals include aggregation and sex pheromones, as well as host-derived kairomones and repellent volatiles, and can be exploited for both population monitoring and behavioural manipulation (Phillips, 1997; Cox, 2004). Recent reviews have confirmed that semiochemical-based tools remain among the main behaviour-oriented components of stored-product IPM, with applications in monitoring, mass trapping, mating disruption, attract-and-kill and push-pull strategies (Anukiruthika et al., 2025; Athanassiou et al., 2025).

Among these applications, monitoring is still the most widely adopted, since pheromone- and kairomone-baited traps allow early detection of infestations and

improve the spatial and temporal precision of control measures (Phillips, 1997; Cox, 2004). By contrast, mating disruption represents the most advanced direct-control application, particularly against pyralid moths. Recent field-scale studies on *Plodia interpunctella* have shown that pheromone-based mating disruption can significantly reduce moth populations in retail and storage environments, although its effectiveness may become apparent only after prolonged application under complex operational conditions (Campbell et al., 2025).

Among stored-product Coleoptera, sitophilure, the aggregation pheromone of *Sitophilus granarius*, has been exploited for many years in monitoring traps and is still the main attractant used for this species. However, host location in *S. granarius* is not mediated exclusively by pheromones. Before physical contact with the substrate, adult granary weevils are able to detect and respond behaviourally to volatile organic compounds (VOCs) emitted by cereal grains (Germinara et al., 2008).

Several studies have shown that these volatiles may influence the orientation behaviour of the insect. In particular, propionic acid was identified as a repellent and fumigant compound active against both *S. granarius* and *S. oryzae*, suggesting its possible use in push–pull strategies (Germinara et al., 2007). Other studies demonstrated that the volatile profiles of different wheat genotypes can affect the behavioural responses of *S. granarius*, indicating that varietal traits may also contribute to host attractiveness and susceptibility to infestation (Germinara et al., 2018). More recently, D’Isita et al. (2024) showed that both *S. granarius* and *Rhyzopertha dominica* respond differently to the VOC profiles of old and modern wheat genotypes, confirming the importance of host-derived chemical cues in insect orientation.

Semiochemical responses in *S. granarius* are not restricted to intact grain kernels. Trematerra et al. (2021) demonstrated that volatiles emitted by cereal- and legume-based macaroni pasta also elicit significant behavioural responses in adults, suggesting that chemical stimuli involved in host location are maintained even in processed food products.

Besides attractants, repellent semiochemicals may also be used as preventive tools. Germinara et al. (2015) showed that some aliphatic aldehydes can interfere with the orientation of *S. granarius* adults towards wheat kernels, reducing the attractiveness of the host substrate. Moreover, Germinara et al. (2020) demonstrated that the application

of inert dusts to grain can interfere with the chemical signals guiding host location and substrate evaluation, thus complementing their physical action with a behavioural disruption effect.

2.4 Botanicals

Among the non-chemical alternatives, plant-derived powders, extracts and essential oils have attracted considerable attention as potential “green” insecticides and repellents for stored-product pests. They align well with circular economy principles when derived from agro-industrial by-products, and they are generally perceived as more acceptable by consumers concerned about synthetic pesticide residues (Pavela, 2016; Hikal et al., 2017; Campolo et al., 2018). Botanical insecticides typically contain complex mixtures of secondary metabolites such as terpenoids, phenylpropanoids, alkaloids, saponins and sterols, which may act singly or synergistically to affect insect survival, behaviour and physiology (Spochacz et al., 2018; Souto et al., 2021). Their bioactivity can manifest as acute toxicity, repellence, antifeedant effects, oviposition deterrence, growth inhibition, or interference with detoxification and endocrine systems (Isman, 2015; Paredes-Sánchez et al., 2021; Rajashekar et al., 2025; Wang et al., 2025). The precise modes of action (MOA) of many botanical compounds are not fully elucidated, but several neurophysiological and metabolic targets have been proposed. For essential oils and various terpenoids, evidence points to interactions with octopaminergic and GABAergic systems, modulation of ion channels, and inhibition of acetylcholinesterase and other key enzymes, leading to hyperexcitation, paralysis and death (Jankowska et al., 2017; Isman, 2015). Non-volatile extracts rich in phenolics, alkaloids or sterols may act by interfering with nutrient assimilation, digestive enzyme activity, or sterol metabolism, thereby affecting growth, moulting and reproduction (Hikal et al., 2017; Ajaha et al., 2019; Singh et al., 2021; Hussein et al., 2023).

A key feature of many botanicals is their multi-target MOA: because they contain numerous active compounds, they can act at several physiological levels simultaneously (Regnault-Roger et al., 2012). This complexity is generally considered to reduce the risk of resistance evolution relative to single-site synthetic insecticides, although systematic resistance studies in stored-product pests are still scarce (Pavela, 2016; Hikal et al., 2017). Despite promising laboratory results, the translation of botanicals into

commercial products for stored-product protection has been relatively slow. Major obstacles include variability in raw material composition due to plant genotype, growing conditions and processing methods, the instability of active compounds, and difficulties in ensuring consistent efficacy under commercial storage conditions (Şuteu et al., 2020). To address these issues, considerable research has focused on advanced formulations and delivery systems. Nano- and microencapsulation techniques using polymers, lipids or cyclodextrins have been developed to enhance the stability, water-dispersibility and controlled release of EOs and plant extracts (Kah et al., 2013; Ayllón-Gutiérrez et al., 2024). Encapsulated botanicals can provide more sustained vapour release, improved adherence to grain or packaging surfaces, and reduced organoleptic impacts, all of which are critical for stored-product applications (Giunti et al., 2023; Maurya et al., 2024; Sarmah et al., 2024). Another promising approach is the combined use of botanicals with other low-impact tools, such as inert dusts or entomopathogens. Several studies have reported synergistic or additive interactions between EOs and diatomaceous earths, allowing lower application rates of each component while maintaining or enhancing efficacy (Islam et al., 2010; Pierattini et al., 2019; Bianchi et al., 2025; Wakil et al., 2025).

Essential oils

Essential oils (EOs) are complex mixtures of volatile compounds, primarily mono- and sesquiterpenes and their oxygenated derivatives obtained by steam distillation, hydrodistillation or cold pressing of aromatic plant tissues (Bakkali et al., 2008; Ayllón-Gutiérrez et al., 2024). They are lipophilic, highly volatile, and often possess a characteristic odour. EOs have been extensively studied as fumigants, contact insecticides and repellents against a wide array of stored-product insects, including *Sitophilus* spp., *Tribolium castaneum*, *Rhyzopertha dominica*, *Ephestia kuehniella* and *Plodia interpunctella* (Isman, 2000; Campolo et al., 2018; Rotundo et al., 2019; Germinara et al., 2017; Al-Harbi et al., 2021). Many EOs exhibit multi-modal activity: they can kill adults and immature stages, deter oviposition, and reduce feeding (Regnault-Roger et al., 2012; Isman, 2019; Chaudhari et al., 2021). Their fumigant

properties make them particularly interesting for the protection of stored grains and packaged products, where vapours can penetrate intergranular spaces and small crevices (Erdoğan & Mustafa, 2021; El-Raheem et al., 2022; Li et al., 2025).

The biological activity of EOs is usually governed by a few major components present at high concentrations such as eugenol, thymol, carvacrol, linalool, 1,8-cineole, and limonene, although minor constituents and synergistic interactions can significantly modulate their overall effects (Dhifi et al., 2016; Mohamed & Alotaibi, 2022; Karabörklü & Ayvaz, 2023).

For example, citrus EOs rich in limonene have shown strong fumigant and repellent effects against several stored-product pests, whereas clove, thyme and oregano EOs, dominated by phenolic monoterpenes, often display potent contact toxicity and feeding deterrence.

From a toxicological standpoint, many EOs and their components have relatively low mammalian toxicity, rapid environmental degradation, and minimal persistence in food and soil, which make them attractive from a regulatory and consumer perspective (Regnault-Roger et al., 2012; Pavela & Benelli, 2016; Al-Harbi et al., 2021). However, their high volatility and sensitivity to light and temperature can also limit their residual efficacy, necessitating appropriate formulation strategies.

Plant extracts

Plant extracts are typically obtained through solvent-based procedures that exploit differences in polarity to selectively isolate distinct classes of secondary metabolites. The most widely employed protocols include maceration in cold or warm solvent, Soxhlet extraction under continuous heating, and more recently, advanced techniques such as ultrasound-assisted and microwave-assisted extraction, which can improve yields and reduce extraction times without significantly altering the chemical profile of the resulting material (Chemat et al., 2017; Chuo et al., 2020; Lefebvre et al., 2020; Cao et al., 2025). An additional approach is supercritical fluid extraction using carbon dioxide, particularly suitable for the selective recovery of non-polar compounds such as terpenoids, allowing the production of solvent-free extracts (Khaw et al., 2017; Uwineza & Waśkiewicz, 2020; Abbas et al., 2026). Within this framework, solvent selection remains a key methodological factor, as it determines which compound classes are

recovered. Compounds are preferentially solubilised in solvents of similar polarity, which defines the distribution of secondary metabolites between the plant matrix and the extraction phase (Harborne, 1998; Nawaz et al., 2020; Sun et al., 2025). For instance, polar solvents such as water, methanol and ethanol preferentially extract phenolic compounds, flavonoids, tannins, alkaloids and polar glycosides, whereas solvents of intermediate polarity such as ethanol-water mixtures and acetone partition both polar and moderately lipophilic compounds, including many saponins, phenylpropanoids and some terpenoid glycosides (Harborne, 1998; Plášková & Mlček, 2023; Tourabi et al., 2025). Non-polar solvents such as hexane, diethyl ether and chloroform, by contrast, selectively solubilise terpenoids, sterols, lipids and waxes (Harborne, 1998; Sun & Tompkinson, 2003; Loneman et al., 2017). This dependency on solvent selection has clear biological implications, as the toxic, antifeedant and deterrent activities observed in plant extracts are closely linked to the extraction protocol employed. As a result, the same plant species may yield extracts with markedly different chemical compositions and bioactivity profiles when different solvents are used (Truong et al., 2019; Onyebuchi & Kavaz, 2020; Kaczorova et al., 2021). For this reason, comparative multi-solvent approaches, in which the same plant material is extracted using solvents with different polarity, have become increasingly important. These approaches make it possible to relate the biological activity of each fraction to specific chemical classes and to better understand the mechanisms underlying the observed effects, going beyond the limits of whole-extract bioassays (Germinara et al., 2011; Rotundo et al., 2019; Paventi et al., 2021).

Beyond the choice of solvent, extraction yield and reproducibility are modulated by numerous additional variables, including the plant organ used, the degree of pulverisation of the starting material, the solid-to-solvent ratio, extraction temperature and duration, and the number of successive extraction steps performed (Gil-Martín et al., 2021; Ramesh et al., 2024). These variables determine the concentration and chemical diversity of bioactive compounds in the extract and affect the reliability and reproducibility of the final product. Moreover, the lack of standardised extraction protocols remains a major limitation in the field of botanical insecticides. Differences in plant material, solvent systems and bioassay conditions often make it difficult to establish whether variations in activity reflect true biological properties or are simply

the result of methodological differences (Pavela et al., 2016; Mathew et al., 2023). In this perspective, the development of shared quality criteria, including the identification and quantification of marker compounds and the definition of minimum effective concentrations, represents a necessary step towards the development and regulatory acceptance of plant-extract-based protectants (Matyjaszczyk, 2023; Rajashekar et al., 2025).

Botanical sources for stored-product insect

Studies on plant extract activity against stored-product insects have encompassed a wide range of families, including Meliaceae, Lamiaceae, Lauraceae, Fabaceae, Piperaceae, Asteraceae, and Liliaceae (Pakeerathan et al., 2021; Ahmed et al., 2021; Akbar et al., 2022; Hasan et al., 2023; Alhoqail, 2025). Within the Lauraceae, extracts and powders of *Cinnamomum verum* have demonstrated significant contact toxicity, repellency and feeding deterrence against major coleopteran pests of stored cereals, including *Sitophilus granarius*, *Rhyzopertha dominica* and *Cryptolestes ferrugineus* (Stephens), with marked differences in susceptibility among target species (Mahmoud et al., 2023).

The genus *Piper* (Piperaceae) constitutes another exceptionally well-documented source of bioactive extracts for stored-product protection. In particular, *Piper nigrum*, the source of commercial black pepper, contains a diverse range of secondary metabolites, including alkaloids (mainly piperine and its analogues), lignans, amides and essential oil components, which are implicated in the insecticidal and antifeedant activity reported against *Sitophilus* spp., *Tribolium castaneum* and *Callosobruchus maculatus* (Fabricius) (Emeribe et al., 2015; Kumar et al., 2024). Moreover, extracts of *P. retrofractum* fruits have been shown to affect adult emergence and fecundity in *Callosobruchus* species and *S. zeamais*, confirming the genus as a rich source of structurally diverse bioactive compounds (Pumnuan et al., 2022).

Among the most investigated botanical sources for stored-product entomology, neem (*Azadirachta indica*, Meliaceae) is of scientific and applied importance. Neem seed kernel extracts are rich in limonoid tetranortriterpenoids, among which azadirachtin, particularly azadirachtin A, is responsible for most of the biological activity (Makanjuola, 1989; Jenkins et al., 2003; Odeyemi & Ashamo, 2005). In stored-product insects, these extracts have been reported to exert repellent and toxic effects, as observed in *Cryptolestes ferrugineus*, *Sitophilus oryzae* and *Tribolium castaneum* (Xie et al.,

1995a). They also affect feeding and reproduction in *Rhyzopertha dominica* (Rahim, 1998) and have been shown to reduce population levels and disrupt growth and development in species such as *Acanthoscelides obtectus*, *Sitophilus oryzae*, *Oryzaephilus surinamensis* and *Cryptolestes ferrugineus* (Facknath, 2006).

The closely related species *Melia azedarach* (Meliaceae) shares a broadly analogous limonoid chemistry with neem, including compounds of the nimbolinin, toosendanin and sendanin types, which have been documented to exert antifeedant and growth-disrupting activity against a range of stored-product Coleoptera and Lepidoptera (Xie et al., 1995b; El-Araby et al., 2014; Ali et al., 2021).

Beyond the Meliaceae, *Humulus lupulus* (Cannabaceae) has emerged as a relevant source of bioactive extracts for stored-product protection. Hop cones contain prenylated flavonoids, bitter acids and polyphenols, which contribute to their biological activity. Paventi et al. (2021) showed that extracts obtained with solvents of different polarity exhibit distinct bioactivity profiles against *S. granarius*, with non-polar fractions displaying stronger contact toxicity and antifeedant effects, and polar fractions showing higher anticholinesterase activity. In addition, electrophysiological assays indicated that hop-derived compounds are detected by the insect olfactory system, suggesting a combined toxic and behavioural mode of action.

Mode of action

Plant extracts affect stored-product insects through multiple mechanisms that extend beyond the neurophysiological targets typically associated with essential oils (Shaaya & Kostyukovsky, 2006; Campolo et al., 2018). While volatile components such as terpenes and phenylpropanoids mainly act on octopaminergic receptors, GABA-mediated signalling and acetylcholinesterase (Jankowska et al., 2017), non-volatile fractions mainly interact with a broader range of physiological processes (Luo et al., 2023; Mobolade, 2023).

Among these mechanisms, plant extracts have been shown to inhibit digestive enzymes (Nathan et al., 2006; Jbilou et al., 2008). Phenolic-rich extracts from plants such as *Punica granatum*, *Rhus coriaria*, *Artemisia sieberi* and *Thymus vulgaris* have been reported to inhibit α -amylase activity in several stored-product insects, with effects depending on both insect species and extract composition (Mehrabadi et al., 2011).

Moreover, phenolic-rich plant extracts are also associated with repellency, contact toxicity and antifeedant activity against stored-product insect pests (Bachrouh et al., 2025; Bączek et al., 2025).

In addition to enzyme inhibition, tannins can impair nutrient assimilation by forming complexes with digestive enzymes and dietary proteins, thereby reducing the availability of assimilable nutrients and negatively affecting development and reproduction (Wongo, 1998). Flavonoids may also influence feeding behaviour through interactions with gustatory receptors, acting as deterrents at higher concentrations or, in some cases, as phagostimulants at lower doses, highlighting the complexity of dose-dependent responses (Nenaah, 2014; Jackowski et al., 2017). Finally, some plant extracts can affect reproductive processes by interfering with oviposition, reducing egg hatchability and increasing the proportion of non-fertile eggs in coleopteran pests of stored products (Spochacz et al., 2018).

3. Agro-industrial by-products and circular economy

The Food and Agriculture Organization estimates that nearly one-third of all food produced globally is lost or wasted along the food supply chain (FAO, 2019; Nicastro & Carillo, 2021).

Fruit and vegetable processing generates millions of tonnes of by-products each year, including peels, pomace, seeds, husks, bracts and leaves (Zhu et al., 2023). Such residues often contain high concentrations of phytochemicals including phenolic acids, tannins, flavonoids, terpenes, essential oils, carotenoids, alkaloids, vitamins, minerals, structural polysaccharides, and fibres that can serve as natural antioxidants, antimicrobials, emulsifiers, stabilisers, gelling agents, nutraceutical ingredients, or functional additives across various industries (Sagar et al., 2018; Bhardwaj et al., 2022; 'Aqilah et al., 2023; Carvalho et al., 2025).

In recent years, beyond their technological and nutritional applications, the biological activity of these compounds has also attracted attention for their potential use in pest management. For instance, citrus peel waste, generated in enormous volumes as a primary by-product of the juice and beverage processing industry, has been among the most extensively investigated matrices. The peel oils and polyphenol-rich extracts from

orange, lemon, and grapefruit processing residues have exhibited significant fumigant and contact toxicity against several stored-product insect, including *Sitophilus* spp., *T. castaneum*, and *R. dominica*, with limonene and other monoterpene constituents of the peel identified as principal bioactive compounds (Campolo et al., 2014; Oboh et al., 2017; Achimón et al., 2022; Nodola et al., 2024). Moreover, rice husk and melon shells have been studied in ash or powder form for the control of stored-product pests (Olorunmota et al., 2017; Otitodun et al., 2017).

Finally, among the most widely cultivated crops in Mediterranean countries, *Punica granatum* L. (pomegranate) and *Cynara scolymus* L. (globe artichoke) generated a lot of residues from the processing industries.

3.1 Pomegranate (*Punica granatum* L.) fruit by-products

The pomegranate, *Punica granatum* L. (pomegranate) is a member of the family Lythraceae (subfamily Punicoideae). The genus *Punica* includes two recognised species, namely *Punica granatum* L., the common pomegranate, and *Punica protopunica* Balf., the Socotran pomegranate (Cronquist, 1981; Guerrero-Solano et al., 2020; Kumari et al., 2021). Historically, the genus *Punica* was classified within the separate family Punicaceae, however, molecular phylogenetic analyses have since confirmed its placement within Lythraceae (Youssef et al., 2018). The pomegranate is native to the region spanning from Iran to northern India, but cultivated across the entire Mediterranean basin, the Middle East, Central Asia, and increasingly in subtropical regions of North and South America, South Africa, and China (Valero-Mendoza et al., 2022). Major producing countries currently include India, Iran, Turkey, Tunisia, Spain, and Morocco, with global production exceeding 3 million tonnes annually (Kahramanoglu, 2019).

The pomegranate fruit is a leathery-skinned berry, botanically classified as a balausta, consisting of a thick, lignified pericarp, comprising the outer exocarp and inner mesocarp, enclosing multiple seed-bearing locules separated by membranous septa (arillate partitions). Each seed is surrounded by a juicy, translucent aril, which constitutes the primary edible fraction consumed fresh or processed into juice, concentrate, molasses, and dried products (Chandra et al., 2010; Benedetti et al., 2023). In industrial processing for juice and concentrate production, the pericarp, commonly

referred to as pomegranate peel, accounts for approximately 45-55% of total fresh fruit weight and is generated as the primary solid by-product (Magangana et al., 2020). In addition to peel, the processing chain generates seed residues following aril pressing, constituting a further 10-15% of fruit weight and retaining substantial lipid and phenolic fractions (Benedetti et al., 2023). Collectively, non-edible fractions represent the majority of the fresh fruit weight processed by industries (Viuda-Martos et al., 2010; Mphahlele et al., 2016).

Among pomegranate by-products, the peel is the fraction most frequently investigated for its phytochemical richness. The total phenolic content values reported in the literature range from 1500 to over 6000 mg GAE/100 g dry weight, with this remarkable variability attributable to cultivar, geographical origin, harvest maturity, and extraction conditions (Li et al., 2006; Tehranifar et al., 2011). The phenolic profile is dominated by hydrolysable tannins, principally ellagitannins, among which punicalagin (α and β isomers) represents the most abundant and structurally distinctive compound, accounting in some cultivars for more than 50% of the total phenolic fraction (Seeram et al., 2006).

The hydrolysis of punicalagin and related compounds including punicalin, pedunculagin, tellimagrandin I and II, and granatin A and B results in ellagic acid and gallic acid, both of which are independently bioactive (Bensaad et al., 2017; Kyriakoudi et al., 2024). The flavonoid fraction includes catechin, epicatechin, quercetin, luteolin, kaempferol, and their glycosides, contributing additional antioxidant and enzyme-inhibitory capacity (Newman et al., 2007; Mphahlele et al., 2016).

Beyond polyphenols, peel extracts contain triterpenoids, notably ursolic acid and oleanolic acid, and phytosterols including β -sitosterol, campesterol, and stigmasterol, which have been associated with membrane-disrupting and growth-regulatory biological activities (Yassin et al., 2021; Sun et al., 2021; El-Aguel et al., 2022). The seed fraction, by contrast, is particularly rich in punicic acid and fatty acid with documented anti-inflammatory and antiproliferative properties, as well as phenolic acids and lower concentrations of ellagitannins (Yamini et al., 2023; Wang et al., 2024).

The complex phytochemical composition of pomegranate fruit is associated with antimicrobial activity against bacterial and fungal pathogens, through mechanisms including membrane disruption, protein precipitation and interference with microbial

enzyme systems (Al-Zoreky, 2009; Gullon et al., 2016). These bioactive constituents have also been associated with antifungal activity against postharvest pathogens, likely related to the inhibition of hyphal growth and spore germination by ellagitannin fractions (Patel et al., 2017; Nair et al., 2018). Finally, compounds derived from pomegranate peel have been shown to exhibit insecticidal, repellent and antifeedant effects (Gupta et al., 2017; Salem et al., 2023; Heba, 2025).

3.2 Artichoke (*Cynara scolymus* L.) by-products

The artichoke, *Cynara cardunculus* L. belongs to the family Asteraceae (Compositae), tribe Cynareae, and represents one of the most economically significant vegetable crops of Mediterranean origin within this family. The species encompasses three botanical varieties: the wild cardoon (*Cynara cardunculus* var. *sylvestris* Lam.), the cultivated cardoon (*Cynara cardunculus* var. *altilis* DC.), and the globe artichoke (*Cynara cardunculus* var. *scolymus* (L.) Fiori), the latter being the principal taxon of agricultural and agro-industrial relevance (Rottenberg & Zohary, 1996; Sonnante et al., 2007). Earlier taxonomic treatments classified the globe artichoke as a distinct species, *Cynara scolymus* L., but molecular phylogenetic evidence has consistently supported its repositioning as a domesticated variety of *C. cardunculus*, likely derived from the wild cardoon through centuries of human selection in the western Mediterranean region (Gatto et al., 2013).

The genus *Cynara* comprises approximately ten species distributed across the Mediterranean basin, the Canary Islands, and western Asia, with the centre of diversity concentrated in the Iberian Peninsula and North Africa (Lombardo et al., 2010).

The globe artichoke is a perennial herbaceous plant reaching 1.5-2 metres in height, characterised by deeply lobed, silver-green leaves, a highly branched stem, and a large capitulum, the harvested inflorescence head, composed of a fleshy receptacle, edible inner bracts, and inedible outer bracts enclosing the immature florets (Lanteri & Portis, 2007; Alicandri et al., 2023). The cultivation is concentrated in Italy, Spain, Egypt, Argentina, and Morocco, with Italy historically accounting for the largest share of global production; world output currently exceeds 1.5 million tonnes of capitula per year (FAO, 2022). In particular, the globe artichoke production in Italy is largely concentrated in the province of Foggia (Apulia, Southern Italy), with an output of around 0.064 Mt,

representing 17% of the national total and approximately 4% of global production (Rana et al., 2023).

The artichoke processing industry generates by-product fractions that account for an extraordinarily high proportion of harvested biomass. Estimates consistently indicate that between 60% and 85% of the fresh capitulum weight is discarded during industrial processing, comprising primarily outer bracts, inner bract trimmings, stems, leaves, and receptacle residues (Corrias et al., 2024).

Among these fractions, the outer bracts, which enclose the capitulum and are characterised by a tough, fibrous and pigmented structure, constitute the largest and most consistently generated sub-fraction, being discarded during the initial stage of industrial trimming (Noriega-Rodríguez et al., 2020).

Traditionally, outer bracts have been destined for composting or landfill disposal, resulting in both management costs and environmental concerns related to their high phenolic content and rapid microbial degradation (Colombo et al., 2024). However, this phenolic richness makes them the most phytochemically valuable fraction of artichoke processing residues, supporting their growing interest in valorisation research (Lavecchia et al., 2018; Jiménez-Moreno et al., 2019; López-Salas et al., 2021).

The phytochemical profile of artichoke outer bracts is dominated by hydroxycinnamic acid derivatives, principally mono- and di-caffeoylquinic acids. The most abundant phenolic compound is the chlorogenic acid (5-*O*-caffeoylquinic acid) followed by cynarin (1,3-di-*O*-caffeoylquinic acid), 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, and 3,4-di-*O*-caffeoylquinic acid (Ayuso et al., 2024; Olas, 2024). The flavonoid fraction contributes additional phytochemical complexity through the presence of luteolin and apigenin glycosides, principally luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside, and apigenin-7-*O*-glucoside, whose concentrations vary substantially with genotype and environmental conditions (Caponio et al., 2025).

A third chemically distinctive class present in artichoke by-products is the sesquiterpene lactones, of which cynaropicrin is the most abundant and biologically characterised representative, accompanied by grosheimin, dehydrocynaropicrin, and aguerin B (Masci et al., 2024).

The antimicrobial activity has been extensively documented for artichoke bract and leaf extracts against both Gram-positive and Gram-negative bacteria and fungal species

(Gaafar & Salama, 2013; Shallan et al., 2020; Ali et al., 2021). By contrast, direct evidence on the insecticidal activity of artichoke by-product extracts against insects remains limited. However, their major phytochemicals have been associated with biological activities relevant to insect control.

For instance, chlorogenic acid and its isomers have been shown to inhibit digestive enzyme function in phytophagous insects, acting as potent inhibitors of midgut α -amylase and proteases, thereby impairing nutrient assimilation and larval development (War et al., 2013; Lin et al., 2022; Punia et al., 2023). Moreover, cynaropicrin and related sesquiterpene lactones have been identified as potentially insect-active compounds. Their chemical structure, characterised by the α -methylene- γ -butyrolactone group, enables interaction with enzymatic systems, a property that has been linked to insecticidal and antifeedant effects reported for structurally related compounds in other plant species (Cis et al., 2006; Sosa et al., 2018; Sülsen & Martino, 2018; Fraga et al., 2021; Evelyn et al., 2024).

4. Aim of the thesis

The development of sustainable pest management strategies for stored-product insects is increasingly recognised as a priority within integrated pest management (IPM) frameworks (Tamilarasan et al., 2025). Whilst a growing body of research has investigated the insecticidal potential of plant-derived compounds, relatively few studies have systematically evaluated extracts obtained directly from agro-industrial by-products of Mediterranean crop species against economically important stored-product pests. In this context, the thesis was mainly focused on characterising the insecticidal, repellent, and antifeedant properties of extracts from pomegranate (*Punica granatum* L.) peel and globe artichoke (*Cynara cardunculus* var. *scolymus*) outer bracts against *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), with the aim to identify: 1) the most bioactive extracts obtained with solvents of different polarity through contact and ingestion toxicity bioassays, repellency assays, and evaluation of nutritional indices; 2) differences in the biological profiles of the two plant matrices; 3) the chemical constituents responsible for the observed activity through a bioassay-guided fractionation approach combining column chromatography, preparative TLC, and GC–MS analysis.

Additionally, during the mandatory research period abroad, a study was carried out to evaluate the influence of temperature, food availability, and dose on the efficacy of λ -cyhalothrin against *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae).

In the framework of current IPM approaches, multidisciplinary activities were also planned and conducted. In particular, studies were carried out to evaluate: 1) the behavioural responses of *S. granarius* and *Rhyzopertha dominica* to volatile organic compounds (VOCs) emitted by old and modern wheat genotypes; 2) the susceptibility and attractiveness of ancient wheat varieties to the same species; 3) the insecticidal activity and long-term persistence of inert dusts, including natural zeolites and diatomaceous earth, against *S. granarius* on stored wheat; 4) the biochemical responses induced by zeolite exposure in *S. granarius*, with particular reference to oxidative stress and the glyoxalase pathway.

5. Insecticidal and repellent activity of different pomegranate peel extracts against granary weevil adults

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Article

Insecticidal and Repellent Activity of Different Pomegranate Peel Extracts Against Granary Weevil Adults

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Simple Summary

The bioactivity of pomegranate peel extracts, obtained by different polarity solvents (acetone, diethyl ether, and *n*-hexane), was evaluated on adults of the granary weevil, *Sitophilus granarius* (L.), in toxicity and repellency tests. The acetone extract showed the strongest toxicity, significantly reduced feeding activity, and exhibited high contact repellency. Chemical analysis revealed sitosterol as the main component. These results support the valorization of pomegranate peel as a natural resource for sustainable stored-product pest management.

Abstract

The pomegranate (*Punica granatum* L.) fruit peel is an agro-industrial by-product rich in bioactive compounds. In this study, the bioactivity of pomegranate peels (cv. Ako) extracted with acetone, diethyl ether, and *n*-hexane was assessed by evaluating toxic (contact and ingestion), repellent, antifeedant, and nutritional effects towards *Sitophilus granarius* (L.) (Coleoptera, Curculionidae) adults. Contact toxicity assays revealed significant mortality induced by the acetone and *n*-hexane extracts, with 24-h LD₅₀ values of 76.93 and 81.14 µg/adult, respectively. In ingestion bioassays, at the highest dose tested (750 µg/disk), the acetone pomegranate peel extract showed a strong feeding deterrence (FDI: 80%), and significantly reduced food consumption (RCR) and relative growth rate (RGR). In filter paper repellency assays, the acetone extract induced positive contact repellency, with PR values ranging from 80% to 30%. GC-MS analysis identified sitosterol, 9,12-octadecadienoic acid, and α -tocopherol as the major constituents of the acetone extract. These results highlight the potential of pomegranate peel as a sustainable source of bioactive compounds for stored-product insect pest management.

Keywords: pomegranate; bioassays; storage pests; bioactive compounds; bioinsecticides



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1. Introduction

In the contemporary era, one of the main global priorities is to ensure a sustainable and efficient food system by increasing production and implementing effective loss-reduction strategies, as the population is expected to reach 9 billion by 2050 [1]. Post-harvest pest infestations in storage facilities represent a major threat to the quality and safety of agricultural commodities [2]. Cereals, a staple of human nutrition, are particularly vulnerable to insect

attacks, which result in considerable economic, and nutritional losses. It is estimated that annually, between 10–20% of global cereal production is lost due to insect infestations when storage conditions are poorly managed [3–5]. Traditionally, stored-grain pest management has relied extensively on contact residual insecticides (e.g., organophosphates, carbamates, and pyrethroids) and fumigants (e.g., phosphine) [6,7]. However, the prolonged and widespread use of these chemicals has raised significant environmental and public health concerns, including the emergence of resistant insect populations, increased pesticide residues in food supplies, and ecological contamination [8]. Consequently, the urgent need for sustainable pest control strategies has driven extensive research towards plant-based formulations, including botanical extracts and essential oils, as alternatives to conventional pesticides, which have been primarily explored under laboratory conditions [9–13]. Among the natural resources being explored, industrial by-products, generated in substantial quantities during food processing [14,15], demonstrated antifungal, antimicrobial, and insecticidal properties [16–21]. Pomegranate (*Punica granatum* L., Lythraceae), a fruit native to Central Asia and widely cultivated for its nutritional and health benefits [22], generates substantial amounts of waste during large-scale food processing. However, the fruit peel, accounting for approximately half of the total weight [23], is rich in bioactive compounds including polyphenols, flavonoids, and tannins [24,25]. These secondary metabolites have been extensively studied for their antioxidant, antimicrobial, and antifungal properties [26–29]. Despite these properties, the bioactivity of pomegranate peel extracts towards insect species has been little investigated. Peel extracts exhibited significant insecticidal activity against the desert locust, *Schistocerca gregaria* (Forsk.) affecting adult performance and biochemical markers like transaminase activity [30]. Moreover, pomegranate extracts showed strong acaricidal effects against house dust mites [31] and effectively managed larvae and adult stages of the cotton leaf worm (*Spodoptera littoralis* (Boisduval)) [32]. Additionally, sublethal toxic effects were observed on the mite pest *Tetranychus urticae* Koch [33], as well as on the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) [34]. Despite this expanding body of literature, to the best of our knowledge, limited research has focused specifically on stored-product insects. The granary weevil, *Sitophilus granarius* (Linnaeus) (Coleoptera: Curculionidae), is among the most damaging pests affecting stored cereal grains, particularly wheat. Its infestation results in substantial direct grain loss through consumption, and secondary damage due to mold development and increased moisture levels [35–37]. Currently, control strategies against *S. granarius* predominantly involve synthetic insecticides, mainly phosphine, organophosphates and pyrethroids, whose resistance issues and detrimental effects on human health and the environment reinforce the necessity for alternative sustainable approaches [38,39]. In this context, this study investigates the insecticidal and repellent activity of different pomegranate peel extracts against *S. granarius* adults. By leveraging the bioactive properties of this by-product, the research aims to contribute to the development of sustainable pest management solutions for stored cereals.

2. Materials and Methods

2.1. Insect Rearing

Adults of *S. granarius* (L.) were reared for six generations at the Department of Agriculture, Food, Natural Resources and Engineering of the University of Foggia. Insects were maintained on wheat grains (*Triticum aestivum* L.) in cylindrical glass containers (Ø 15 × 15 cm) closed with a metallic net (0.5 mm). The rearing was maintained in the dark at 25 ± 2 °C and 60 ± 5% relative humidity (r.h.). Newly emerged adults were collected daily and maintained under the same rearing conditions. Unsexed adult beetles, 3- to 4-weeks old, were used only once per experiment.

2.2. Plant Material

Pomegranate fruits (*Punica granatum* L.) (Ako cultivar) were collected in mid-September 2022 from organic farms located in Foggia (Italy) (GPS coordinates: 41.101740) at the full ripeness stage. The fertilization regimen included the application of 100 units of phosphorus at the start of vegetative growth, 150 units of nitrogen during the vegetative phase, and 150 units of potassium before fruit maturation.

2.3. Pomegranate Peels Extracts

Pomegranate peels (3.71 kg) were manually removed from different fruits. Peels were oven-dried at 27 ± 2 °C for 15 days until constant weight, reaching a total dry weight of 0.94 kg (corresponding to a 74.7% reduction in fresh weight). A low-temperature, forced-air drying process was adopted to preserve the chemical integrity and bioactivity of the plant material [40,41]. The dried peels were ground using an SM 100 mill (Retsch, Haan, Germany; 2001) and the resulting powder was sieved (0.5 mm mesh). Pomegranate peel material (100 g) was soaked in acetone, diethyl ether, or *n*-hexane solvents (300 mL) for 24 h at room temperature, kept in the dark, and gently hand-mixed four times for 5 min. The mixtures were then centrifuged (40,000 rpm, 10 min, T 10 °C), and the supernatant was filtered (Whatman No. 113) (Cytiva, Marlborough, MA, USA). Solvents were then removed under vacuum using a rotary evaporator (40 °C, 200 mbar) (Laborota 4000, Heidolph, Schwabach, Germany). Each extraction was repeated twice to ensure procedural consistency. The residues obtained from *n*-hexane, diethyl ether, and acetone extracts, respectively, of 1.4 ± 0.4 , 4.2 ± 0.6 , 40.8 ± 12.3 g/kg dry weight, were stored at -20 °C.

2.4. Contact Toxicity Bioassay

The contact toxicity of different pomegranate peel extracts against *S. granarius* adults was assessed through topical application [42,43]. The extracts were dissolved in their respective solvents (acetone, diethyl ether and *n*-hexane), and two-fold serial dilutions were prepared (150.00 to 18.75 µg/µL). A 0.5 µL aliquot of each dilution was directly applied onto the pronotum of *S. granarius* adults in a temporary state of immobility (thanatosis) (700 series, Microliter™, Hamilton Company, Reno, NV, USA). Each concentration was tested on three groups of 10 unsexed adults. Replicates were set up on the same day under identical laboratory conditions. Insects treated with solvent alone were used as controls. For each replicate, insects treated ($n = 10$) were transferred in a Petri dish and positioned within a metal ring (Ø 4.0 × 2.5 cm) covered with a metallic mesh (1 mm) to prevent escape. Five wheat kernels were provided to insects as food. The entire setup was kept in darkness at 26 ± 2 °C and $60 \pm 5\%$ relative humidity. Mortality rate was recorded after 24 and 48 h of extracts application. Individuals were considered dead when showing no movement even after gentle stimulation with a fine hair camel brush. The recorded mortality percentages were arcsine square-root transformed and submitted to one-way ANOVA analysis followed by Tukey's HSD test for means comparison. Data normality (Shapiro–Wilk test) and variance homogeneity (Levene's test) were assessed before analysis. The lethal doses required to achieve 50% and 90% mortality (LD₅₀ and LD₉₀), alongside their upper and lower confidence limits, regression equations, and chi-square (χ^2) values, were calculated through probit analysis [44]. Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) Version 23 for Windows (SPSS Inc., Chicago, IL, USA).

2.5. Ingestion Toxicity, Antifeedant, and Nutritional Activity

The effect of different pomegranate peel extracts on the feeding and nutritional indices of adult granary weevils was evaluated by the flour disk bioassay, following the method

of Xie (1996) [45] with minor modifications [43]. Wheat flour (2.5 g) was gently mixed with distilled water (10 mL) using magnetic stirring. Flour disks were then obtained by depositing 100 µL aliquots of the mixture onto plastic Petri dishes which were subsequently left to dry overnight (26 ± 2 °C and $60 \pm 5\%$ r.h.) ensuring a consistent moisture content of the disks [46]. Flour disks were then treated with 5 µL of each extract solution, corresponding to 93.75, 187.50, 375.00, 750.00 µg/disk concentrations. Disks treated with solvent alone were used as control. To allow solvent evaporation, both treated and solvent-control disks were maintained at room temperature for 2 h before insect exposure. In a pre-weighed glass vial ($\varnothing 2.5 \times 4.0$ cm), two treated flour disks were placed, and their weight was recorded. Subsequently, ten group-weighed weevil adults were introduced into each vial, which was then re-weighed and maintained in the darkness (26 ± 2 °C, $60 \pm 5\%$ r.h.) for three days. Post-experiment, the number of dead insects was recorded for each vial, and the weight of both the remaining flour disks and the surviving insects were separately determined. For each extract concentration and control, 5 replicates, each encompassing 10 insects, were set up on the same day. The following nutritional indices [47,48] were calculated for each replicate:

$$\text{Relative Growth Rate (RGR)} = \frac{A - B}{B} \text{ day}^{-1} \quad (1)$$

$$\text{Relative Consumption Rate (RCR)} = \frac{D}{B} \text{ day}^{-1} \quad (2)$$

$$\text{Efficiency Conversion of Ingested Food (ECI)} = \frac{\text{RGR}}{\text{RCR}} \times 100 \quad (3)$$

$$\text{Feeding Deterrence Index (FDI)} = \frac{C - T}{C} \times 100 \quad (4)$$

where A is the mean weight (mg) of live insects on the third day; B is the initial mean weight (mg) of insects; C is the consumption of control disks; D is the biomass ingested (mg) per number of living insects on the third day; and T indicates the consumption of treated disks. Data normality (Shapiro–Wilk test) and variance homogeneity (Levene's test) were assessed before performing ANOVA, followed by post hoc comparisons using Tukey's HSD test.

2.6. Repellency Bioassay

The repellent activity of pomegranate peel extracts was evaluated using the area preference redmethod [43,49]. A filter paper disc (Whatman No. 1, $\varnothing 8.0$ cm, area = 50.2 cm²) was divided in half. One half was treated with 500 µL of pomegranate peel extract solution using a micropipette, while the other half was treated with an equal volume of the solvent alone as a control. Both the treated and control halves were air-dried for approximately 10 min to allow complete evaporation of the solvent. The two halves were then joined with transparent adhesive tape, and the full disc was fixed to the bottom of a Petri dish ($\varnothing 9.0$ cm). Ten unsexed adult weevils were confined on each filter paper disc within a metal O-ring ($\varnothing 8.0 \times 4.0$ cm) covered with a metallic mesh (1 mm) to prevent escape. The experiment was conducted in darkness at 26 ± 2 °C and $60 \pm 5\%$ r.h. Solutions of pomegranate peel extracts, prepared as described in Section 2.3, were tested at concentrations of 0.37, 0.75, 1.49, and 2.98 mg/cm², respectively. For each extract concentration 4 replicates, each encompassing 10 insects, were set up on the same day. The number of weevils on the treated (N_t) and control (N_c) portions of the paper disc was recorded after 10 min, 30 min, 1 h, 2 h, and 24 h. The percent repellency (PR) of each extract was calculated using the following formula:

$$\text{Percent Repellency (PR) (\%)} = \frac{(N_c - N_t)}{(N_c + N_t)} \times 100 \quad (5)$$

where N_c is the number of insects present in the control half paper and N_t the number of insects present in the treated one. Data normality and variance homogeneity were verified using the Shapiro–Wilk and Levene tests, respectively. PR values were subjected to repeated-measures ANOVA, with Greenhouse–Geisser correction applied when Mauchly’s test of sphericity was violated. Means at each exposure time were separated by Tukey’s HSD test ($p < 0.05$).

2.7. Gas Chromatography–Mass Spectrophotometry (GC-MS) Analysis

The crude acetone extract of pomegranate peels was diluted 1:100 with acetone and a 1 μ L sample was injected in the gas chromatographic system. A 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with an Agilent 5977 mass selective detector (MSD) and equipped with a HP-5MS capillary column (30 m $\times$ 0.25 mm i.d., 0.5 μ m film thickness, Agilent Technologies, Santa Clara, CA, USA) was used. The carrier gas was helium at a flow rate of 1.25 mL/min. The injection was made in the splitless mode, and the injector temperature was 250 $^{\circ}$ C. The oven temperature was initially held at 100 $^{\circ}$ C for 5 min then programmed to 280 $^{\circ}$ C at 6.0 $^{\circ}$ C/min with a final holding time of 40 min. Spectra were recorded in the electron impact mode (ionization energy, 70 eV) in the range of 35–550 amu at 2.9 scans/s. A solvent delay time of 5 min was used to avoid overloading the mass spectrometer with the solvent. The identification of compounds was achieved by comparing mass spectra with those of the data system library (NIST11). Moreover, a mixture of a continuous series of straight-chain hydrocarbons, C8–C20 (Alkane Standard Solution C8–C20, Sigma Aldrich, Milan, Italy) was injected into the HP-5MS column under the same conditions previously described for the acetone peels extract to calculate the linear retention indices (RIs), according to the method of van den Dool and Kratz (1963) [50]. A compound was considered identified when the spectral similarity exceeded 90% and the experimental RI corresponded to the RI reported in the literature (NIST Chemistry WebBook or scientific sources for the HP-5MS column) within ± 10 units. Component relative percentages were calculated based on GC peak areas. Due to the known limitations of non-polar columns such as the HP-5MS in separating β -, γ -, and α -sitosterol isomers, the peak detected at RI 3203 was assigned to an isomeric mixture of sitosterols, as previously reported [51–53].

3. Results

3.1. Contact Bioassay

Mortalities of *S. granarius* adults 24 and 48 h after topical application of different pomegranate peel extracts are presented in Tables 1–3. Among all extracts, the strongest contact toxicity was shown by acetone and *n*-hexane extracts whereas the diethyl ether extract exhibited a low toxicity. After 24 h treatment, acetone and *n*-hexane extracts induced significantly higher mortalities than control (ANOVA: acetone, $F = 25.6$, $df = 4$, $p < 0.001$; *n*-hexane, $F = 52.9$, $df = 4$, $p < 0.001$) starting from the 37.5 μ g/adult dose whereas no significant mortality ($F = 0.50$, $df = 4$, $p = 0.737$) was recorded for diethyl ether extract at any dose tested. The 24 h LD₅₀ values calculated for the *n*-hexane and acetone extracts were 81.14 and 76.93 μ g/adult, respectively. These values slightly decreased 48 h after treatment (Tables 1–3).

Table 1. Contact toxicity of different concentrations of the *n*-hexane extract of pomegranate peel against adults of *S. granarius* after 24 and 48 h from topical application. For each exposure time, mean mortality values followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey's HSD test).

Dose ($\mu\text{g}/\text{Adult}$)	Exposure Time (h)	% Mortality (Mean $\pm$ S.E.)	Regression Equation	χ^2	LD ₅₀ (95% CL, $\mu\text{g}/\text{Adult}$)	LD ₉₀ (95% CL, $\mu\text{g}/\text{Adult}$)
150	24 h	73.33 $\pm$ 3.33 a	$y = -4.24 - 2.22x$	0.487	81.14 (62.47–114.80)	306.37 (188.26–826.73)
75		43.33 $\pm$ 3.33 b				
37.5		26.67 $\pm$ 3.33 b				
18.75		6.67 $\pm$ 3.33 c				
0		0.00 $\pm$ 0.00 c				
F		52.9				
d.f.		4				
<i>p</i>		<0.001				
150	48 h	76.67 $\pm$ 3.33 a	$y = -4.44 - 2.36x$	0.315	75.68 (59.06–102.96)	264.08 (170.50–619.14)
75		46.67 $\pm$ 6.67 ab				
37.5		26.67 $\pm$ 3.33 b				
18.75		6.67 $\pm$ 3.33 c				
0		0.00 $\pm$ 0.00 c				
F		57.5				
d.f.		4				
<i>p</i>		<0.001				

Table 2. Contact toxicity of different concentrations of the acetone extract of pomegranate peel against adults of *S. granarius* after 24 and 48 h from topical application. For each exposure time, mean mortality values followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey's HSD test).

Dose ($\mu\text{g}/\text{Adult}$)	Exposure Time (h)	% Mortality (Mean $\pm$ S.E.)	Regression Equation	χ^2	LD ₅₀ (95% CL, $\mu\text{g}/\text{Adult}$)	LD ₉₀ (95% CL, $\mu\text{g}/\text{Adult}$)
150	24 h	76.67 $\pm$ 8.82 a	$y = -4.84 - 2.57x$	0.938	76.93 (60.95–102.14)	231.76 (161.52–243.08)
75		46.67 $\pm$ 12.02 ab				
37.5		26.67 $\pm$ 8.82 b				
18.75		3.33 $\pm$ 3.33 c				
0		0.00 $\pm$ 0.00 c				
F		25.6				
d.f.		4				
<i>p</i>		<0.001				
150	48 h	83.33 $\pm$ 6.67 a	$y = -4.74 - 2.61x$	0.362	65.31 (51.87–84.35)	202.45 (140.44–392.09)
75		53.33 $\pm$ 8.82 ab				
37.5		30.00 $\pm$ 5.77 bc				
18.75		6.67 $\pm$ 3.33 cd				
0		0.00 $\pm$ 0.00 d				
F		34.7				
d.f.		4				
<i>p</i>		<0.001				

Table 3. Contact toxicity of different concentrations of the diethyl ether extract of pomegranate peel against adults of *S. granarius* after 24 and 48 h from topical application. For each exposure time, mean mortality values followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey's HSD test).

Dose ($\mu\text{g}/\text{Adult}$)	Exposure Time (h)	% Mortality (Mean $\pm$ S.E.)	Regression Equation	χ^2	LD ₅₀ (95% CL, $\mu\text{g}/\text{Adult}$)	LD ₉₀ (95% CL, $\mu\text{g}/\text{Adult}$)
150	24 h	3.33 $\pm$ 3.33 a	$y = -3.08 - 0.63x$	0.831	83,757.38	9,332,208.94
75		3.33 $\pm$ 3.33 a				
37.5		3.33 $\pm$ 3.33 a				
18.75		0.00 $\pm$ 0.00 a				
0		0.00 $\pm$ 0.00 a				
F		0.50				
d.f.		4				
<i>p</i>		0.737				
150	48 h	10.00 $\pm$ 5.77 a	$y = -3.86 - 1.25x$	1.185	1212.40	12,772.71
75		10.00 $\pm$ 0.00 a				
37.5		3.33 $\pm$ 3.33 a				
18.75		0.00 $\pm$ 0.00 a				
0		0.00 $\pm$ 0.00 a				
F		3.63				
d.f.		4				
<i>p</i>		0.045				

3.2. Ingestion Toxicity, Antifeedant, and Nutritional Activity

The ingestion toxicity, antifeedant, and nutritional effects of pomegranate peel extracts towards *S. granarius* adults are summarized in Tables 4–6. Overall, mortality was low across all treatments. The acetone extract induced a significant dose-dependent reduction in relative growth rate (RGR, $F = 9.25$; $df = 4$; $p = 0.002$), relative consumption rate (RCR, $F = 9.05$; $df = 4$; $p = 0.002$) and efficiency of conversion of ingested food (ECI, $F = 14.94$; $df = 4$; $p < 0.01$). In contrast, the *n*-hexane and diethyl ether extracts elicited no appreciable variation in these parameters, with values remaining statistically comparable to those of the corresponding controls ($p > 0.05$). The feeding deterrence index (FDI) showed a significant dose-dependent increase exclusively for the acetone extract, reaching the highest value of 75.13% at the highest concentration ($F = 40.57$; $df = 4$; $p < 0.001$) tested. In contrast, diethyl ether and *n*-hexane extracts exhibited positive but non-significant FDI values across all concentrations ($F = 1.3$ – 1.1 , $df = 4$, $p > 0.05$).

Table 4. Nutritional indices, mortality and food deterrence of *S. granarius* adults exposed to different concentrations of the *n*-hexane extract of pomegranate peel. Means in the same column with the same letter are not significantly different at the 0.05 level determined by Tukey's HSD test.

Concentration (µg/Disk)	Mortality (%)	RGR (mg/mg/Day) (Mean ± S.E.)	RCR (mg/mg/Day) (Mean ± S.E.)	ECI (%) (Mean ± S.E.)	FDI (%) (Mean ± S.E.)
750.00	26.00 a	−0.018 ± 0.009 a	0.089 ± 0.034 a	−19.709 ± 11.770 a	32.444 ± 10.691 a
375.00	20.00 ab	−0.016 ± 0.012 a	0.091 ± 0.006 a	−19.806 ± 13.195 a	34.467 ± 1.931 a
187.50	14.00 ab	−0.011 ± 0.013 a	0.091 ± 0.014 a	−17.215 ± 13.836 a	30.791 ± 10.090 a
93.75	2.00 b	0.007 ± 0.010 a	0.113 ± 0.018 a	−12.119 ± 11.842 a	10.778 ± 14.826 a
Control	0.00 b	0.010 ± 0.005 a	0.125 ± 0.014 a	6.504 ± 4.353 a	

Table 5. Nutritional indices, mortality and food deterrence of *S. granarius* adults exposed to different concentrations of the acetone extract of pomegranate peel. Means in the same column with the same letter are not significantly different at the 0.05 level determined by Tukey's HSD test.

Concentration (µg/Disk)	Mortality (%)	RGR (mg/mg/Day) (Mean ± S.E.)	RCR (mg/mg/Day) (Mean ± S.E.)	ECI (%) (Mean ± S.E.)	FDI (%) (Mean ± S.E.)
750.00	20.00 a	−0.063 ± 0.006 a	0.071 ± 0.012 a	−97.566 ± 15.177 a	75.131 ± 3.467 a
375.00	14.00 ab	−0.066 ± 0.005 a	0.088 ± 0.009 a	−79.050 ± 11.455 a	66.728 ± 2.424 ab
187.50	10.00 ab	−0.060 ± 0.002 a	0.112 ± 0.010 ab	−56.152 ± 7.901 ab	56.056 ± 4.806 b
93.75	6.00 ab	−0.057 ± 0.115 a	0.136 ± 0.013 b	−31.950 ± 9.887 bc	13.298 ± 5.779 c
Control	0.00 b	0.010 ± 0.006 b	0.144 ± 0.006 b	4.886 ± 4.353 c	

Table 6. Nutritional indices, mortality and food deterrence of *S. granarius* adults exposed to different concentrations of the diethyl ether extract of pomegranate peel. Means in the same column with the same letter are not significantly different at the 0.05 level determined by Tukey's HSD test.

Concentration (µg/Disk)	Mortality (%)	RGR (mg/mg/Day) (Mean ± S.E.)	RCR (mg/mg/Day) (Mean ± S.E.)	ECI (%) (Mean ± S.E.)	FDI (%) (Mean ± S.E.)
750.00	16.00 a	−0.002 ± 0.009 a	0.094 ± 0.018 a	−17.864 ± 23.316 a	32.333 ± 14.221 a
375.00	22.00 a	−0.005 ± 0.010 a	0.101 ± 0.017 a	−9.635 ± 7.827 a	31.750 ± 15.803 a
187.50	12.00 ab	−0.004 ± 0.006 a	0.095 ± 0.008 a	−3.140 ± 7.563 a	33.800 ± 3.878 a
93.75	0.00 b	0.002 ± 0.007 a	0.128 ± 0.015 a	−0.096 ± 5.510 a	6.000 ± 9.176 a
Control	0.00 b	0.008 ± 0.010 a	0.132 ± 0.008 a	4.936 ± 6.971 a	

3.3. Area Preference Bioassay

The repellent activity of pomegranate peel extracts against *S. granarius* adults was evaluated using filter paper disc bioassays. Among the extracts tested, the acetone extract was the most effective, particularly at the highest dose (2.98 mg, $F = 6.24$, $df = 3$, $p < 0.05$), achieving a PR of 80% after 10 min of exposure (Table 7). In contrast, the *n*-hexane and diethyl ether extracts exhibited markedly lower and less consistent repellent effects (Tables 8 and 9). Statistical analysis confirmed a significant effect of dose ($F = 15.76$; $p < 0.001$) and time ($F = 6.24$; $p < 0.001$), with no significant dose × time interaction ($p = 0.300$) (Table 10) for acetone extract. The *n*-hexane extract showed a significant effect of

dose ($F = 5.05$; $p = 0.017$), whereas the diethyl ether extract did not show any significant effect with dose ($F = 1.37$; $p = 0.300$), time ($F = 0.30$; $p = 0.827$), or their interaction ($F = 1.57$; $p = 0.132$) (Tables 11 and 12).

Table 7. Percent repellency (PR) ($\pm$ S.E.) of different concentrations of the *n*-hexane extract of pomegranate fruit peels against *S. granarius* adults in filter paper disc bioassays after different exposure times. Values in the same column followed by different letters are significantly different at $p < 0.05$ (Tukey HSD test).

Dose (mg/cm ²)	Exposure Time				
	10 min	30 min	60 min	120 min	24 h
2.98	45.00 $\pm$ 9.57 a	45.00 $\pm$ 9.58 a	25.82 $\pm$ 12.91 a	50.00 $\pm$ 12.91 a	30.00 $\pm$ 12.91 a
1.49	25.00 $\pm$ 9.57 ab	25.00 $\pm$ 15.00 ab	20.00 $\pm$ 8.16 a	20.00 $\pm$ 21.60 a	50.00 $\pm$ 12.91 a
0.75	−25.00 $\pm$ 9.57 bc	−25.00 $\pm$ 9.57 bc	5.00 $\pm$ 20.61 a	5.00 $\pm$ 20.61 a	5.00 $\pm$ 20.61 a
0.37	−20.00 $\pm$ 14.14 c	−15.00 $\pm$ 5.77 b	10.00 $\pm$ 20.82 a	5.00 $\pm$ 5.00 a	5.00 $\pm$ 17.08 a
F	9.9	8.7	1.5	1.7	1.8
d.f.	3	3	3	3	3
<i>p</i>	0.000	0.000	0.000	0.000	0.000

Table 8. Percent repellency (PR) ($\pm$ S.E.) of different concentrations of the acetone extract of pomegranate fruit peel against *S. granarius* adults in filter paper disc bioassays after different exposure times. Values in the same column followed by different letters are significantly different at $p < 0.05$ (Tukey HSD test).

Dose (mg/cm ²)	Exposure Time				
	10 min	30 min	60 min	120 min	24 h
2.98	80.00 $\pm$ 8.16 a	55.00 $\pm$ 9.58 a	55.00 $\pm$ 5.00 a	30.00 $\pm$ 12.91 a	30.00 $\pm$ 12.91 a
1.49	60.00 $\pm$ 8.16 ab	40.00 $\pm$ 8.16 a	40.00 $\pm$ 8.16 ab	20.00 $\pm$ 8.16 a	20.00 $\pm$ 8.16 a
0.75	30.00 $\pm$ 10.00 bc	25.00 $\pm$ 15.00 a	−5.00 $\pm$ 20.61 b	−10.00 $\pm$ 12.91 a	−10.00 $\pm$ 12.91 a
0.37	5.00 $\pm$ 12.58 c	−30.00 $\pm$ 5.77 b	−5.00 $\pm$ 12.58 b	−10.00 $\pm$ 12.91 a	−10.00 $\pm$ 12.91 a
F	11.13	13.20	5.70	3.00	3.00
d.f.	3	3	3	3	3
<i>p</i>	0.0009	0.0004	0.0118	0.0728	0.0728

Table 9. Percent repellency (PR) ($\pm$ S.E.) of different concentrations of the diethyl ether extract of pomegranate fruit peels against *S. granarius* adults in filter paper disc bioassays after different exposure times. Values in the same column followed by different letters are significantly different at $p < 0.05$ (Tukey HSD test).

Dose (mg/cm ²)	Exposure Time				
	10 min	30 min	60 min	120 min	24 h
2.98	10.00 $\pm$ 20.82 a	−5.00 $\pm$ 15.00 ab	10.00 $\pm$ 20.82 a	10.00 $\pm$ 17.32 a	15.00 $\pm$ 12.58 a
1.49	−25.00 $\pm$ 15.00 bc	10.00 $\pm$ 5.77 a	−25.00 $\pm$ 17.08 a	−25.00 $\pm$ 17.08 a	15.00 $\pm$ 15.00 a
0.75	15.00 $\pm$ 12.58 bc	−10.00 $\pm$ 12.90 ab	5.00 $\pm$ 17.08 a	5.00 $\pm$ 17.08 a	10.00 $\pm$ 17.32 a
0.37	−20.00 $\pm$ 16.33 c	−45.00 $\pm$ 15.00 b	−15.00 $\pm$ 15.00 a	−15.00 $\pm$ 17.32 a	−15.00 $\pm$ 20.61 a
F	1.6	4.8	0.9	1.1	0.7
d.f.	3	3	3	3	3
<i>p</i>	0.000	0.000	0.000	0.000	1.000

Table 10. Repeated measures analysis of variance between subjects effects for the repellent activity of the acetone extract of pomegranate fruit peels against *S. granarius* adults in filter paper disc bioassays at the doses of 2.98, 1.49, 0.75, and 0.37 mg/cm² after 10, 30, 60, 120 min and 24 h exposure, respectively.

Source	df	Mean Square	F-Value	<i>p</i> -Value
Dose	3	17,258.333	15.761	<0.001
Error	12	1095		
Exposure time	4	2542.500	6.239	<0.001
Error	12	311.667		
Dose $\times$ Exposure time	12	495.833	1.217	0.300
Error	48	407.500		

Table 11. Repeated measures analysis of variance between subjects effects for the repellent activity of the *n*-hexane extract of pomegranate fruit peel against *S. granarius* adults in filter paper disc bioassays at the doses of 2.98, 1.49, 0.75, and 0.37 mg/cm² after 10, 30, 60, 120 min and 24 h exposure, respectively.

Source	df	Mean Square	F-Value	p-Value
Dose	3	9773.333	5.046	0.017
Error	12	1936.667		
Exposure time	4	607.500	0.948	0.445
Error	12	1305		
Dose × Exposure time	12	527.500	0.823	0.626
Error	48	640.833		

Table 12. Repeated measures analysis of variance between subjects effects for the repellent activity of the diethyl ether extract of pomegranate fruit peels against *S. granarius* adults in filter paper disc bioassays at the doses of 2.98, 1.49, 0.75, and 0.37 mg/cm² after 10, 30, 60, 120 min and 24 h exposure, respectively.

Source	df	Mean Square	F-Value	p-Value
Dose	3	3885	1.366	0.300
Error	12	2845		
Exposure time	4	129.167	0.298	0.827
Error	12	434.167		
Dose × Exposure time	12	739.167	1.573	0.132
Error	48	470		

3.4. Gas Chromatography–Mass Spectrophotometry (GC-MS) Analysis

The main components of the pomegranate peel acetone extract and their peak area (%) are presented in Table 13. The extract chemical profiling revealed the presence of 10 compounds corresponding to 95% of the total extract. Squalene (3.63%), 5-hydroxymethylfurfural (7.34%), α -tocopherol (9.82%), hexadecanoic acid (12.29%), 9,12-octadecadienoic acid (25.14%), and sitosterol (34.46%) along with 4 other minor compounds with relative peak areas ranging from 0.44 to 0.77% were identified.

Table 13. Chemical composition of acetone pomegranate peel extract.

Peak No.	Compound	¹ R.T. (min)	² RI _{Exp}	³ RI _{Lit}	Area (%)
Hydrocarbons and Terpenes					
2	Tetradecene	10.92	1423	1425	0.63
3	trans- α -Bergamotene	11.94	1586	1584	0.44
5	(3E)-Elisocene	15.30	2022	2020	0.51
Total Hydrocarbons and Terpenes					1.58
Lipids					
6	Hexadecanoic acid	22.11	1966	1964	12.29
7	9,12-Octadecadienoic acid	24.84	2089	2086	25.14
8	Squalene	33.68	2818	2820	3.63
9	α -Tocopherol	37.49	3132	3130	9.82
10	Sitosterols (isomeric mixture)	40.72	3200	3203	34.46
Total Lipids					85.34
Others					
1	5-Hydroxymethylfurfural	7.38	1235	1233	7.34
4	2,4-Di-tert-butylphenol	13.74	1602	1605	0.77
Total Others					8.11

¹ R.T. = retention time in minutes; ² RI_{Exp} = Determined linear retention index against a mixture of *n*-alkanes (C₅–C₄₀) on HP-5MS column; ³ RI_{Lit} = Linear retention index from literature.

4. Discussion

Among fruit by-products, pomegranate peels are particularly rich in bioactive compounds [54]. This study indicates that pomegranate peel extracts possess insecticidal potential against *S. granarius* adults, depending on the solvent used for extraction (acetone, *n*-hexane, and diethyl ether). Notably, in contact toxicity bioassays, the acetone and

n-hexane extracts achieved the highest mortality rates (70–77%), contrasting with the very low toxicity observed for the diethyl ether extract (3%). Comparable results have been reported for extracts from other botanical matrices [55,56], underscoring the crucial role of solvent selection in optimizing the recovery of insecticidal bioactive compounds.

The contact toxicity of the acetone pomegranate peel extract against the granary weevil in this study ($LD_{50} = 76.93 \mu\text{g}/\text{adult}$) was lower than that reported for the acetone extract from *Humulus lupulus* (L.) aerial parts ($LD_{50} = 16.17 \mu\text{g}/\text{adult}$) [43], but higher than that of the acetone extract from *Sideritis trojana* (Bornm.) ($LD_{50} = 136.60 \mu\text{g}/\text{adult}$) [57]. The contact toxicity of the *n*-hexane pomegranate peel extract ($LD_{50} = 81.14 \mu\text{g}/\text{adult}$) was markedly lower than that of *Melia azedarach* (L.) ($LD_{50} = 1.7 \mu\text{g}/\text{adult}$) [58] but comparable to that of *Dirtrichia viscosa* (L.) ($LD_{50} = 53.2 \mu\text{g}/\text{adult}$) [42].

Considering other stored-product insect pests, pomegranate peel ethanolic extracts have exhibited strong contact toxicity against *Tribolium castaneum* (Herbst) larvae and adults [59]. Similarly, El-Sayed and Said (2017) [60] reported strong contact insecticidal effects of a methanolic pomegranate peel extract against *Callosobruchus maculatus* (Fabricius) adults, with an LD_{50} of 1.22%. The contact toxicity observed in this study is therefore consistent with previous findings on other pest species. For instance, El Sakaan et al. (2024) [61] observed significant larval mortality in *S. littoralis* after treatment with ethanolic pomegranate peel extracts, while Jung (2015) [31] reported 100% mortality of house dust mites following exposure to pomegranate peel extracts prepared with the same solvent.

In ingestion bioassays, the acetone extract also significantly affected both nutritional and growth parameters of the granary weevil, in contrast to the *n*-hexane and diethyl ether extracts. Most notably, at the highest concentration tested (750 $\mu\text{g}/\text{disk}$), the acetone extract exhibited a strong feeding deterrence (75%). No antifeedant effect was found for extracts from *Scrophularia canina* (L.) or *D. viscosa* against the same pest [42,62]. Moreover, the high FDI value of the acetone extract contrasts with the low antifeedant effects observed for other plant-based extracts against stored-product insect pests. For instance, a methylene chloride extract of *Cinnamomum aromaticum* (Nees) induced only 35% feeding deterrence in *Sitophilus zeamais* (Motschulsky) adults and near-zero FDI values in *T. castaneum* larvae and adults [63]. Furthermore, Haridasan et al. (2017) [64] reported an FDI of about 66% for *Vitex negundo* (L.) leaf extracts towards *T. castaneum*. The antifeedant activity of the acetone pomegranate peel extract in this study also aligns with previous findings on *S. littoralis* larvae, which showed strong antifeeding responses to ethanol and methanol pomegranate peel extracts (92.96% $\pm$ 2.92 and 77.63% $\pm$ 3.78, respectively) [65].

Our contact repellency assays showed a dose-dependent effect of pomegranate peel extracts, with the acetone extract reaching up to 80% repellency at the highest concentration tested (2.98 mg/cm²). The *n*-hexane extract showed a more moderate repellency, not exceeding 50%. Similar repellent effects against *S. granarius* were also reported for extracts from *H. lupulus* [43] and *Prangos ferulacea* (Lindley) [66]. Interestingly, ethanolic pomegranate peel extracts have produced contrasting results in different species. Mohammad et al. (2012) [67] reported up to 100% contact repellency against *T. confusum* at the highest dose tested (10%), while Hamouda et al. (2014) [59] observed limited repellency ($\leq 20\%$) against *T. castaneum* at a 2% concentration. Such discrepancies suggest that, beyond concentration, the susceptibility to *P. granatum* extracts may vary considerably among insect species.

Repellent materials can provide immediate protection to stored cereals by deterring insects before feeding or oviposition, thereby limiting damage and preventing population growth. Moreover, because repellents do not necessarily kill pests, they exert lower selection pressure, potentially reducing the likelihood of resistance development [68,69]. The strong repellency of the pomegranate peels acetone extract observed in this study could be used to complement integrated pest management strategies by combining repellent and

attractive compounds, according to a push-pull approach, to manipulate insect behaviour and distribution. In addition, this extract could be applied to prevent insect infestation in storage facilities or used as a chemical barrier to protect packaged food products from insect invasion [70,71].

The GC-MS analysis of the acetone extract revealed sitosterol (isomeric mixture) as one of the major constituents, accounting for approximately 34.46% of the total extract. This phytosterol is widely distributed in plants, and its biological activity against several insect pests has been reported. For instance, β -sitosterol may interfere with insect cholesterol metabolism, reducing larval development and adult emergence, as documented in *Helicoverpa armigera* (Hübner) and *Dermestes maculatus* (Fabricius), where its accumulation due to inefficient metabolism leads to toxicity [72,73]. Beyond its physiological effects, β -sitosterol from petroleum ether extract of *Abutilon indicum* (L.) has demonstrated larvicidal activity against *Culex quinquefasciatus* (Say), a mosquito species of medical importance [74]. In addition to sitosterol, other compounds identified in the acetone extract, including squalene, 5-hydroxymethylfurfural (5-HMF), α -tocopherol, hexadecanoic acid, 9,12-octadecadienoic acid, and 2,4-di-tert-butylphenol, have also been reported to exhibit insecticidal activity [75–82].

In conclusion, pomegranate peel is a rich source of bioactive compounds with insecticidal, antifeedant, and repellent activities. However, further studies are required to clarify the role of individual constituents, assess sublethal effects, and validate efficacy under field conditions, as well as to extend evaluations to other insect pests and non-target organisms.

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6. Biological Activity of Pomegranate Peel for the green management of *Ephesia kuehniella* (Lepidoptera: Pyralidae) larvae

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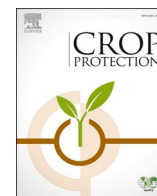
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Research article

Biological activity of pomegranate peel for the green management of *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae

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ABSTRACT

The biological activity of pomegranate peel extracts (acetone, *n*-hexane, and diethyl ether) against larvae of *Ephestia kuehniella* was evaluated through contact, ingestion, and repellency bioassays. In topical application assays, the acetone extract exhibited the highest toxicity, with LD₅₀ values of 140.52 and 131.27 µg/larva after 24 and 48 h, respectively. The *n*-hexane extract displayed intermediate activity, whereas the diethyl ether extract was less effective. In flour disk ingestion assays, the acetone extract significantly impaired larval performance at 750 µg/disk, inducing pronounced feeding deterrence (FDI: 79.99%) and marked reductions in relative growth and consumption rates. In filter paper area preference assays, the acetone extract reached 80% repellency shortly after exposure, compared with lower responses recorded for the *n*-hexane and diethyl ether extracts. Overall, the acetone extract of pomegranate peel, characterized by the predominance of sitosterols, 9,12-octadecadienoic acid, and *n*-hexadecanoic acid, represents a promising candidate for the development of plant-based strategies in stored-product protection.

1. Introduction

The Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), is among the most damaging stored-product insects, causing direct losses through larval feeding on stored grains and flour, and indirect losses due to contamination mainly with silk webbing (Poziđi-Metaxa and Athanassiou, 2013; Aouadi et al., 2020; Kurtuluş et al., 2020; Adamić Zamljen et al., 2025). Conventional management of this pest relies mainly on chemical insecticides, however, their efficacy is influenced by several factors, including developmental stage, insecticide dose, and exposure time. For instance, organophosphates, pyrethroids, and pyrethroids are more effective against younger than older *E. kuehniella* larvae (Stejskal et al., 2021), while the performance of pyrethroids combined with heat treatments depends strongly on both dose and exposure duration (Javanshir et al., 2022). Similarly, the use of fumigants, particularly phosphine, is well established but stage-dependent; although phosphine is highly effective against larvae and pupae, it shows reduced efficacy against eggs, requiring prolonged

exposure for complete control (Bell, 1979; Güncan et al., 2023). In addition, the intensive use of synthetic insecticides has been associated with adverse effects on human health and non-target organisms (Arkan and Turan, 2020; Park et al., 2020; Wan et al., 2025). Collectively, these limitations highlight the urgent need for sustainable alternatives related to *E. kuehniella* management. Several non-chemical strategies have been explored for the control of this pest. These include pheromone-mediated mating disruption, improved storage management to reduce infestation risk, and the use of biological control agents such as entomopathogenic fungi, bacteria, and parasitoids (Athanassiou et al., 2017; Trematerra, 2018; Bahmani et al., 2020; Yaman et al., 2021; Kallaf et al., 2021; Papanikolaou et al., 2021; Yalçın, 2023; Aqeel et al., 2024). Physical control methods, such as the application of extreme temperatures through refrigeration or rapid heating, have also proven effective against different developmental stages of the moth (Andreadis and Athanassiou, 2017). In addition, modified or controlled atmosphere techniques that lower oxygen or increase carbon dioxide concentrations have shown potential in suppressing the *E. kuehniella* populations (Cao

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et al., 2019). Nevertheless, in recent years, increasing attention has been directed toward plant-derived compounds, particularly those recovered from processing residues, as environmentally alternatives for managing stored-product insect pests (Mattos et al., 2021; Visakh et al., 2022; El Kasimi et al., 2023; Sulhath et al., 2024). Among industrial by-products, pomegranate (*Punica granatum* L.) peels are known to contain a wide array of bioactive compounds with diverse biological activities (Barathikannan et al., 2016; Xiang et al., 2022; Siddiqui et al., 2024; Azmat et al., 2024). This part of the fruit is generally regarded as waste and is often discarded by the processing industry. However, the discovery of pomegranate peel multifunctional properties has significantly increased its industrial value. It has been reported to act as a natural additive enhancing food stability (Kaderides et al., 2021), to retard lipid oxidation in tahini (Ghasemi et al., 2023) and serve as a natural antioxidant incorporated into edible coatings or films (Kumar et al., 2022). Moreover, its antimicrobial activity has been exploited in food preservation (Mostafa et al., 2021), in dermatological formulations for human skin (Radan et al., 2024), as well as in the control of several insect species (Zahrán et al., 2024; Fouda et al., 2023; El Sakaan et al., 2024). In the context of *E. kuehniella* management, numerous studies have examined the insecticidal properties of essential oils, demonstrating strong contact, fumigant, and repellent effects (Ercan et al., 2013; Pandir and Baş, 2016; El-Bakry et al., 2024). However, there remains a lack of research on the bioactivity of plant extracts against this species. In our previous work, acetone extracts of pomegranate peels exhibited significant insecticidal and antifeedant effects against *Sitophilus granarius* (L.) adults (Lo Muzio et al., 2025). Building on this evidence, the present study aims to assess the insecticidal potential of pomegranate peel extracts against *E. kuehniella* larvae, contributing to the development of sustainable strategies for stored-product pest management.

2. Materials and methods

2.1. Insects

A laboratory colony of the Mediterranean flour moth was established at the Department of Agriculture, Food, Natural Resources and Engineering (University of Foggia), starting from adults collected from local storage facilities, since 2021. The species was subsequently maintained under laboratory conditions for approximately 40 generations. Larvae were reared on a refined white soft wheat flour (*Triticum aestivum* L., type 00) without additives, in glass cylindrical containers (Ø 15 × 15 cm) sealed with a 0.1 mm metallic mesh. Insect cultures were maintained at 25 °C, 65% relative humidity (RH), and complete darkness. To obtain second instar-larvae, *E. kuehniella* adults were allowed to oviposit on the wheat flour for 3 days. Eight-day-old larvae were selected as second instars based on head capsule width (0.26–0.38 mm), following the morphometric criteria described by Harvey and Thompson (1995) and Harvey and Vet (1997).

2.2. Plant material

The fruit peels of pomegranate were dried at 35 °C for 10 days in a forced-air drying oven (Heratherm OMH750, Thermo Fisher Scientific, Langensfeld, Germany), then ground and sieved through a 0.5 mm mesh (ASTM E11 No. 35; Fritsch GmbH; Idar-Oberstein, Germany). Extracts were prepared using solvents of decreasing polarity, specifically acetone, diethyl ether, and *n*-hexane. For each extraction, 100 g of the ground plant material was soaked in 300 mL of the respective solvent, followed by agitation and a resting period of 24 h at room temperature (25 °C). The mixtures were then washed twice with the same solvents, centrifuged at 40,000 rpm for 10 min at 10 °C, filtered using Whatman No. 113 filters (Cytiva, Marlborough, MA, USA). Solvents were then removed under vacuum using a rotary evaporator (40 °C, 200 mbar) (Laborota 4000, Heidolph, Schwabach, Germany).

2.3. GC-MS analysis

GC–MS was used to characterize the qualitative composition and relative abundance of the acetone extract from pomegranate peel employed in the bioassays. Analyses were carried out on an Agilent 7890B gas chromatograph coupled to an Agilent 5977 mass selective detector (MSD) through a direct capillary connection. Chromatographic separation was achieved on an HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.5 µm film thickness; Agilent Technologies, Santa Clara, CA, USA), using helium as carrier gas at a constant flow rate of 1.25 mL/min. A 1-µL aliquot of the extract, previously diluted 1:100 (v/v) in acetone, was injected in splitless mode at 250 °C, while the MSD interface was maintained at 280 °C. The oven temperature program was as follows: 100 °C for 5 min, increased at 6 °C/min to 280 °C, and held at this temperature for 40 min. Mass spectra were recorded in electron impact mode at 70 eV over the *m/z* range 35–550 at 2.9 scans/s. Data acquisition started after a 5-min solvent delay. Compounds were tentatively identified by comparing their mass spectra with those in the NIST11 library. The relative abundance of each constituent was estimated from the integrated peak area and expressed as relative peak area (RA, %) with respect to the total chromatographic area. Because HP-5MS and other non-polar stationary phases do not allow chromatographic separation of β-, γ- and δ-sitosterol isomers, the peak corresponding to these compounds was conservatively assigned as a sitosterol isomeric mixture, in agreement with the approach reported by Sheng and Chen (2009).

2.4. Contact toxicity

The contact toxicity of pomegranate peel extracts against *E. kuehniella* larvae was evaluated using the topical application method. Extracts were individually dissolved in acetone, *n*-hexane, and diethyl ether, and two-fold serial dilutions were prepared to obtain concentrations ranging from 18.75 to 150.00 µg/µL. An aliquot of 1 µL from each dilution was applied dorsally to the larvae using a Hamilton micro-syringe. For each concentration, three replicates of 10 larvae each (*n* = 30 larvae per concentration) were tested, while control groups received applications of the corresponding solvent alone (Abbassy et al., 2007; Yooboon et al., 2019; Ammar et al., 2025). Treated insects were maintained in darkness at 26 °C and 60% RH. During the experiment, larvae were not provided with food. Mortality was assessed after 24 and 48 h of exposure. Larvae were considered dead when no movement was observed after gentle stimulation with a fine brush for 2 min. The bioassay procedure followed the protocol described by Rotundo et al. (2019) and Paventi et al. (2021). Mortality percentages were arcsine square-root transformed prior to analysis to meet the assumptions of normality and homogeneity of variance. Mortality values, expressed as the mean of three independent replicates (*n* = 10 larvae per replicate), were analyzed using a factorial analysis of variance (ANOVA), with extract, concentration, exposure time (24 or 48 h), and their interactions included as fixed factors. Lethal doses causing 50% and 90% mortality (LD₅₀ and LD₉₀), along with their 95% confidence limits, regression equations, and chi-square (χ^2) values, were estimated by probit analysis (Finney, 1971). Statistical analyses were conducted using SPSS software, version 23.0 (SPSS Inc., Chicago, IL, USA).

2.5. Ingestion toxicity, antifeedant, and nutritional activity

The effects of pomegranate peel extracts on the feeding behaviour and nutritional indices of *E. kuehniella* larvae were evaluated using a flour disk bioassay (Xie et al., 1996). Wheat flour disks were prepared by homogenizing wheat flour (2.5 g) with distilled water (10 mL) under gentle magnetic agitation until a uniform suspension was obtained. Aliquots (100 µL) of the mixture were deposited onto plastic Petri dishes and dried overnight under controlled conditions (26 °C and 60% RH) to obtain disks with uniform size and moisture content. Disks were treated

with 5 μL of extract solutions at concentrations of 93.75, 187.50, 375.00, and 750.00 μg per disk (Rotundo et al., 2019). Control disks were treated with solvent alone. After drying, the disks were placed individually in glass vials containing 10 larvae and maintained under controlled conditions (26 °C and 60% RH) for three days. Five independent replicates were performed per concentration, each consisting of 10 larvae ($n = 50$ larvae per concentration) (Mehrkhou et al., 2013; Yousefi-Lardeh et al., 2025). At the end of the exposure period, larval mortality, food consumption, and insect weight were recorded. Nutritional indices, including the Relative Growth Rate (RGR), Relative Consumption Rate (RCR), Efficiency of Conversion of Ingested Food (ECI), and Feeding Deterrence Index (FDI), were calculated according to standard procedures (Farrar et al., 1989; Huang and Ho, 1998). Data were analyzed separately for each extract by one-way ANOVA, followed by Tukey's HSD test for mean separation.

2.6. Repellence in filter paper bioassay

The contact repellent activity of pomegranate peel extracts was evaluated using the area preference method. Circular filter papers (Whatman No. 1; $\varnothing$ 8.0 cm; area = 50.2 cm^2) were cut in half, having one half treated with 500 μL of the extract solution and the other with an equal volume of solvent alone (control). Both halves were air-dried for approximately 10 min to allow complete solvent evaporation, then reattached with transparent adhesive tape and fixed to the bottom of a Petri dish ($\varnothing$ 9.0 cm). The filter paper disc was confined within a metal O-ring ($\varnothing$ 8.0 $\times$ 4.0 cm) sealed with a fine metallic mesh (1 mm) to avoid larval escape. A group of 10 *E. kuehniella* larvae was gently released at the center of the disc, and the setup was maintained in darkness under controlled environmental conditions (26 °C and 60% RH). Extract solutions were tested at four concentrations (0.37, 0.75, 1.49, and 2.98 mg/cm^2), with four independent replicates per treatment, each consisting of 10 larvae ($n = 40$ larvae per concentration) (Gitahi et al., 2021). The percent repellence (PR) of each extract was calculated by the formula: $\text{PR} (\%) = [(N_c - N_t)/(N_c + N_t)] \times 100$ where N_c is the number of insects present in the control half paper and N_t the number of insects present in the treated one (McDonald et al., 1970). PR values were submitted to repeated measures analysis, with Greenhouse–Geisser correction applied when Mauchly's test of sphericity was violated. For each exposure time, mean PR values were separated by Tukey's HSD test. The bioassay procedure followed the area preference method previously described by Germinara et al. (2017) and Paventi et al. (2021).

3. Results

3.1. GC-MS analysis

GC–MS analysis of the acetone extract from pomegranate peel allowed the tentative identification of ten compounds accounting for

approximately 95% of the total chromatographic area (Table 1). Sitos-terols, detected as an unresolved isomeric mixture, represented the most abundant fraction of the extract (34.54%), followed by 9,12-octadeca-dienoic acid (25.06%) and hexadecanoic acid (12.35%). Appreciable relative amounts of α -tocopherol (9.76%), 5-hydroxymethylfurfural (7.28%), and squalene (3.69%) were also detected. The remaining four compounds occurred only in minor proportions, each contributing less than 1% of the total chromatographic area.

3.2. Contact toxicity

Mortality rates of *E. kuehniella* larvae recorded 24 and 48 h after topical application of pomegranate peel extracts are summarized in Figs. 1 and 2 (mean $\pm$ SE, $n = 30$ larvae per concentration). At 24 h, both acetone and *n*-hexane extracts caused significantly higher mortality compared with the control (acetone: $F(4,10) = 32.5$, $p < 0.001$; *n*-hexane: $F(4,10) = 39.5$, $p < 0.001$), starting from the 37.5 $\mu\text{g}/\text{larva}$. Conversely, for diethyl ether extract, a significant mortality was observed only at the highest dose tested (150 $\mu\text{g}/\text{larva}$) ($F(4,10) = 31 = 30.0$, $p < 0.001$). A similar pattern was observed at 48 h, with significant differences in mortality among treatments for acetone ($F(4,10) = 59.4$, $p < 0.001$) and *n*-hexane extracts ($F(4,10) = 22.6$, $p < 0.001$) while no variation was observed for the diethyl ether extract. The estimated LD_{50} values after 24 h were 140.52 $\mu\text{g}/\text{larva}$ for the acetone extract and 209.37 $\mu\text{g}/\text{larva}$ for the *n*-hexane extract, while the diethyl ether extract displayed an LD_{50} value of 225.94 $\mu\text{g}/\text{larva}$ (Table 2). After 48 h, LD_{50} values decreased slightly for both acetone and

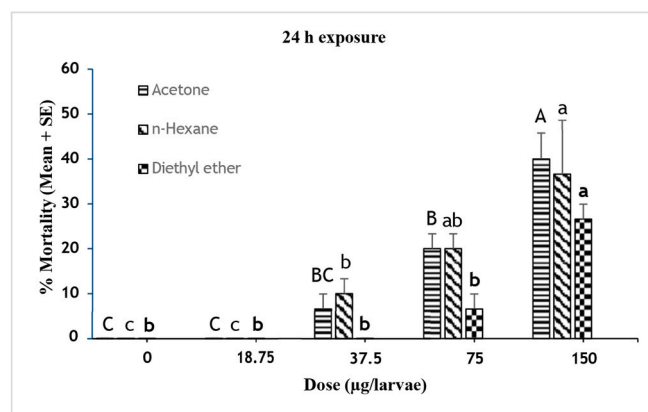


Fig. 1. Contact mortality (mean $\pm$ SE) of *E. kuehniella* larvae to ascending doses of different pomegranate peel extracts after 24 h from exposure. Within each extract, different letters indicate significant differences among doses at $p = 0.05$ (Tukey's HSD test). Data are based on three independent replicates ($n = 30$ larvae per concentration).

Table 1

Compounds identified in acetone extract from *P. granatum* peels by GC/MS.

No.	Compound	Classification	^a RT (min)	Molecular formula	Molecular weight	^b RA (%)
1	5-(Hydroxymethyl)furfural	Furan derivative	7.41	C ₆ H ₆ O ₃	126.11	7.28
2	Tetradecene	Hydrocarbon	10.88	C ₁₄ H ₂₈	196.37	0.66
3	trans- α -Bergamotene	Sesquiterpene	11.98	C ₁₅ H ₂₄	204.35	0.41
4	2,4-Di-tert-butylphenol	Phenolic compound	13.69	C ₁₄ H ₂₂ O	206.32	0.81
5	Hexadecanol	Fatty alcohol	15.34	C ₁₆ H ₃₄ O	242.44	0.47
6	Hexadecanoic acid	Fatty acid	22.06	C ₁₆ H ₃₂ O ₂	256.42	12.35
7	9,12-Octadecadienoic acid	Fatty acid	24.91	C ₁₈ H ₃₂ O ₂	280.45	25.06
8	Squalene	Triterpene	33.72	C ₃₀ H ₅₀	410.72	3.69
9	α -Tocopherol	Vitamin E	37.44	C ₂₉ H ₅₀ O ₂	430.71	9.76
10	Sitosterol isomer(s)	Phytosterol	40.77	C ₂₉ H ₅₀ O	414.71	34.54
Total identified compounds						95.03

Comparison with NIST11 spectral library data.

^a R.T. = retention time in minutes.

^b RA (%) = relative peak area.

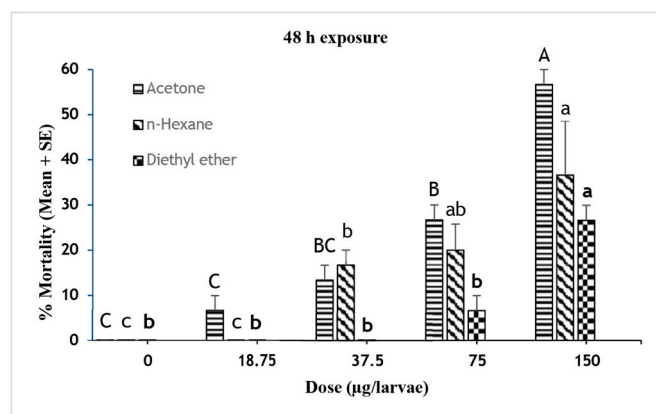


Fig. 2. Contact mortality (mean $\pm$ SE) of *E. kuehniella* larvae to ascending doses of different pomegranate peel extracts after 48 h from exposure. Within each extract, different letters indicate significant differences among doses at $p = 0.05$ (Tukey's HSD test). Data are based on three independent replicates ($n = 30$ larvae per concentration).

n-hexane extracts (131.27 and 195.44 $\mu\text{g/larva}$, respectively), whereas they remained unchanged for the diethyl ether extract. Factorial ANOVA revealed significant effects of extract and concentration on larval mortality, as well as a significant extract $\times$ concentration interaction (Table 3). In contrast, exposure time and its interactions did not significantly affect mortality.

3.3. Nutritional indices

The ingestion toxicity, antifeedant activity, and nutritional responses of *E. kuehniella* larvae to pomegranate peel extracts are reported in Tables 4–6 (mean $\pm$ SE, $n = 50$ larvae per concentration). Mortality remained low across all tested concentrations. A significant dose-dependent decrease in RGR, RCR, and ECI was observed in larvae treated with the acetone extract (Table 4). Moreover, the acetone extract showed a significant concentration effect on Feeding Deterrence Index, with FDI values increasing progressively with concentration and reaching a maximum of 79.99% at 750 $\mu\text{g/disk}$. Conversely, the *n*-hexane (Table 5) and diethyl ether (Table 6) extracts did not significantly influence these nutritional parameters, as their values were comparable with those observed in the control groups ($p > 0.05$).

3.4. Repellence in filter paper bioassay

The three extracts exhibited different levels of contact repellency in

filter paper disc bioassays (Fig. 3), with values expressed as mean $\pm$ SE of four independent replicates ($n = 40$ larvae per concentration). Among them, the acetone extract showed the highest activity, with PR values up to 80% after 10 min. For this extract, repeated measures ANOVA showed a significant effect of dose ($F(3,12) = 4.926$, $p = 0.019$) and exposure time ($F(4,48) = 9.129$, $p < 0.001$), while the dose $\times$ exposure time interaction was not significant ($F(12,48) = 0.866$, $p = 0.553$) (Table 7). For the *n*-hexane extract, neither dose, exposure time, nor their interaction were significant. Similarly, for the diethyl ether extract, dose, exposure time, and their interaction were not significant.

4. Discussion

Due to the increasing global demand for pomegranate juice, that is recognised for its health benefits, large quantities of peel are generated during industrial processing, as only the edible arils are utilized (Hegazi et al., 2021; Tarantino et al., 2022). Peels account for approximately 40–50% of the fruit weight and are characterized by high tannin content, bitterness, and susceptibility to rapid microbial spoilage, which limit their direct industrial application and contribute to their designation as low-value agro-industrial by-products (Mphahlele et al., 2019; Magangana et al., 2020; Belgacem et al., 2021). Despite these limitations, the documented biological activities of pomegranate peel highlight its potential use within sustainable pest management strategies. In this context, the present study evaluated different solvent extracts of pomegranate peel against larvae of *E. kuehniella* through contact, ingestion, and repellence bioassays. Since the biological activity of plant extracts is closely related to their phytochemical composition, acetone, *n*-hexane, and diethyl ether were used to extract different classes of bioactive compounds which may vary in their insecticidal efficacy (Tiwari et al., 2011; Azmir et al., 2013; Kumar et al., 2020). Specifically, acetone is known to recover phenolic and flavonoid constituents (Do

Table 3

Results of factorial analysis of variance (ANOVA) on arcsine square-root transformed mortality of *E. kuehniella* larvae, testing the effects of extract, concentration, exposure time, and their interactions.

Factor	df	F	p
Extract	2, 60	60.94	≤ 0.001
Concentration	4, 60	154.96	≤ 0.001
Exposure time	1, 60	0.33	0.565
Extract $\times$ Concentration	8, 60	8.95	≤ 0.001
Extract $\times$ Exposure time	2, 60	0.08	0.920
Concentration $\times$ Exposure time	4, 60	0.06	0.993
Extract $\times$ Concentration $\times$ Exposure time	8, 60	0.07	1.000

Table 2

Lethal doses 50 (LD₅₀) and 90 (LD₉₀) of the three pomegranate peel solvent extracts after 24 and 48 h of *E. kuehniella* larvae exposure.

Solvent extract	Exposure (h)	^a LD ₅₀ /LD ₉₀ ($\mu\text{g/larvae}$)	^b 95% CL for LD ₅₀	^b 95% CL for LD ₉₀	^c χ^2 (df)	p-value
<i>n</i> -Hexane	24	209.37/932.03	134.70 – 685.89	381.00 – 15110.76	1.08 (4)	<0.001
Acetone	24	140.52/543.56	102.00 – 258.50	184.79 – 2554.53	0.06 (4)	<0.001
Diethyl ether	24	225.94/543.91	159.75 – 970.41	281.68 – 13597.52	0.24 (4)	<0.001
<i>n</i> -Hexane	48	195.44/904.27	127.25 – 587.83	373.91 – 12839.16	1.78 (4)	<0.001
Acetone	48	131.27/511.92	96.18 – 231.72	272.98 – 2230.85	0.24 (4)	<0.001
Diethyl ether	48	225.94/543.91	159.75 – 970.41	281.68 – 13597.52	0.24 (4)	<0.001

^a LD: lethal doses killing 50% (LD₅₀) or 90% (LD₉₀) of the exposed population.

^b CL: confidence limit.

^c χ^2 : chi square.

Table 4

Ingestion mortality, nutritional indices, and food deterrence of *E. kuehniella* larvae of different concentrations of acetone extract. Means in the same column with the same letter are not significantly different at $p = 0.05$ (Tukey's HSD test). Data are based on five independent replicates ($n = 50$ larvae per concentration).

Concentration ($\mu\text{g}/\text{Disk}$)	Mortality (%)	RGR (mg/mg/day) (mean $\pm$ S.E.)	RCR (mg/mg/day) (mean $\pm$ S.E.)	ECI (%) (mean $\pm$ S.E.)	FDI (%) (mean $\pm$ S.E.)
750.00	12.00 a	-0.015 ± 0.011 a	0.094 ± 0.012 a	-20.438 ± 14.314 a	79.988 ± 2.716 a
375.00	14.00 a	0.020 ± 0.009 ab	0.202 ± 0.031 ab	8.219 ± 4.179 ab	56.409 ± 8.549 ab
187.50	8.00 a	0.051 ± 0.012 bc	0.322 ± 0.037 bc	15.948 ± 4.428 b	34.889 ± 7.210 b
93.75	6.00 a	0.072 ± 0.013 bc	0.392 ± 0.027 c	18.301 ± 1.566 b	41.149 ± 3.405 b
Control	0.00 a	0.102 ± 0.017 c	0.398 ± 0.058 c	25.581 ± 2.081 b	
F	1.87 (4,20)	12.74 (4,20)	13.11 (4,20)	6.27 (4,20)	11.18 (3,16)
p	0.154	<0.001	<0.001	0.002	<0.001

Table 5

Ingestion mortality, nutritional indices, and food deterrence of *E. kuehniella* larvae of different concentrations of *n*-hexane extract. Means in the same column with the same letter are not significantly different at $p = 0.05$ (Tukey's HSD test). Data are based on five independent replicates ($n = 50$ larvae per concentration).

Concentration ($\mu\text{g}/\text{Disk}$)	Mortality (%)	RGR (mg/mg/day) (mean $\pm$ S.E.)	RCR (mg/mg/day) (mean $\pm$ S.E.)	ECI (%) (mean $\pm$ S.E.)	FDI (%) (mean $\pm$ S.E.)
750.00	6.00 a	-0.001 ± 0.016 a	0.183 ± 0.030 a	-3.289 ± 11.086 a	44.839 ± 7.729 a
375.00	4.00 a	0.020 ± 0.008 a	0.178 ± 0.014 a	11.827 ± 4.700 a	24.856 ± 3.739 a
187.50	4.00 a	0.033 ± 0.013 a	0.211 ± 0.012 a	14.485 ± 5.650 a	23.300 ± 2.818 a
93.75	8.00 a	0.031 ± 0.011 a	0.212 ± 0.042 a	15.191 ± 1.547 a	12.397 ± 19.434 a
Control	0.00 a	0.036 ± 0.006 a	0.258 ± 0.037 a	16.945 ± 4.186 a	
F	0.512 (4,20)	1.757 (4,20)	1.155 (4,20)	1.316 (4,20)	1.591 (3,16)
P	0.846	0.177	0.360	0.298	0.231

Table 6

Ingestion mortality, nutritional indices, and food deterrence of *E. kuehniella* larvae of different concentrations of diethyl ether extract. Means in the same column with the same letter are not significantly different at $p = 0.05$ (Tukey's HSD test). Data are based on five independent replicates ($n = 50$ larvae per concentration).

Concentration ($\mu\text{g}/\text{Disk}$)	Mortality (%)	RGR (mg/mg/day) (mean $\pm$ S.E.)	RCR (mg/mg/day) (mean $\pm$ S.E.)	ECI (%) (mean $\pm$ S.E.)	FDI (%) (mean $\pm$ S.E.)
750.00	0.00 a	0.010 ± 0.006 a	0.263 ± 0.028 a	3.971 ± 0.628 a	16.082 ± 5.966 a
375.00	0.00 a	0.027 ± 0.010 a	0.202 ± 0.007 a	13.258 ± 1.364 a	20.901 ± 3.936 a
187.50	0.00 a	0.030 ± 0.004 a	0.245 ± 0.007 a	15.761 ± 5.347 a	19.263 ± 2.966 a
93.75	0.00 a	0.030 ± 0.010 a	0.249 ± 0.039 a	11.765 ± 3.068 a	0.761 ± 21.411 a
Control	0.00 a	0.035 ± 0.006 a	0.237 ± 0.021 a	14.614 ± 1.998 a	
F	n.d.	1.549 (4,20)	0.909 (4,20)	1.615 (4,20)	0.655 (3,16)
p	n.d.	0.227	0.477	0.209	0.591

et al., 2014; Iloki-Assanga et al., 2015) reported to have an antifeedant and insecticidal potential (Simmonds, 2003; War et al., 2012). In contrast, the non-polar solvents *n*-hexane and diethyl ether predominantly recover lipophilic compounds, including terpenoids and sterols. Terpenoids have been associated with contact toxicity and effects on insect development and reproduction (Pavela, 2015; Qasim et al., 2024; Abdelgaleil et al., 2022), with evidence suggesting neurophysiological modes of action (Enan, 2001; Kostyukovsky et al., 2002; Jankowska et al., 2017). Sterols, in turn, are essential for insect development, as insects rely on dietary sterols for membrane structure and insect development (Behmer and Nes, 2003). Across all bioassays, solvent controls showed negligible larval mortality, indicating that the observed effects were primarily attributable to the pomegranate peel extracts rather than to the solvents used, which are commonly employed in insect bioassays (Kamaraj et al., 2009; Nair et al., 2014; Adesina et al., 2019; Paventi et al., 2021). In the contact toxicity bioassay, differences in efficacy among the tested extracts were observed. The acetone extract induced the highest mortality rate in *E. kuehniella* larvae and consequently the lowest LD₅₀ value after 48 h of exposure, compared to the *n*-hexane and diethyl ether extracts. In comparison, *Sideritis trojana* Bornm. acetone extract required longer exposure times (96 h) to reach the 40% of mortality in *E. kuehniella* larvae (Aslan et al., 2006). This result may be attributed to the intermediate polarity of acetone, which may have affected the types and relative abundance of bioactive compounds. Indeed, acetone has been shown to extract a broader spectrum of phytochemicals, potentially contributing to enhanced biological activity (Kanmani et al., 2021; Ammar et al., 2025). In addition to contact toxicity, an ingestion bioassay was performed using the same pomegranate peel extracts. Among them, the acetone extract, at the

concentration of 750 $\mu\text{g}/\text{disk}$, induced a significant alteration of normal feeding behaviour, with a deterrent effect reaching 79.99%. Furthermore, a marked impairment of larval growth and metabolism was observed, as shown by the negative values of the nutritional indices considered. This effect is consistent with literature evidence indicating that acetone efficiently extracts flavonoids, tannins, and other phenolic compounds (Dai and Mumper, 2010; Dirar et al., 2019; Kumar et al., 2023; Lee et al., 2024). These metabolites, which are known to occur in pomegranate peel, have been reported to disrupt larval metabolism and feeding behaviour in various insect species (Pavela et al., 2019; Mossa, 2016; Hamouda et al., 2014; D'Isita et al., 2023; El Sakaan et al., 2024). The contact repellent activity toward *E. kuehniella* larvae was elicited by all *P. granatum* peel extracts at the highest concentration tested, with the acetone extract reaching a peak of 80% contact repellency (PR). A similar short-term repellency pattern was previously reported by Hamouda et al. (2014) for *P. granatum* peel extracts obtained with different solvents towards *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) adults. However, a progressive decline in deterrent activity over time was observed for all extracts, which can be attributed to the oxidation or degradation of polyphenolic compounds (Bąkowska et al., 2003; Barbehenn and Constabel, 2011; Belščak-Cvitanović et al., 2018) leading to a reduced availability and perceptibility of active compounds to the larvae. From a practical perspective, this limitation could be overcome by developing more stable formulations, such as microencapsulated or emulsified systems, to extend their residual activity and facilitate their application under real storage conditions (Fang and Bhandari, 2010; Lu et al., 2016; Campolo et al., 2020; Caballero et al., 2022; Li et al., 2024). Pomegranate peel extracts have also been reported to exhibit low mammalian toxicity and rapid environmental

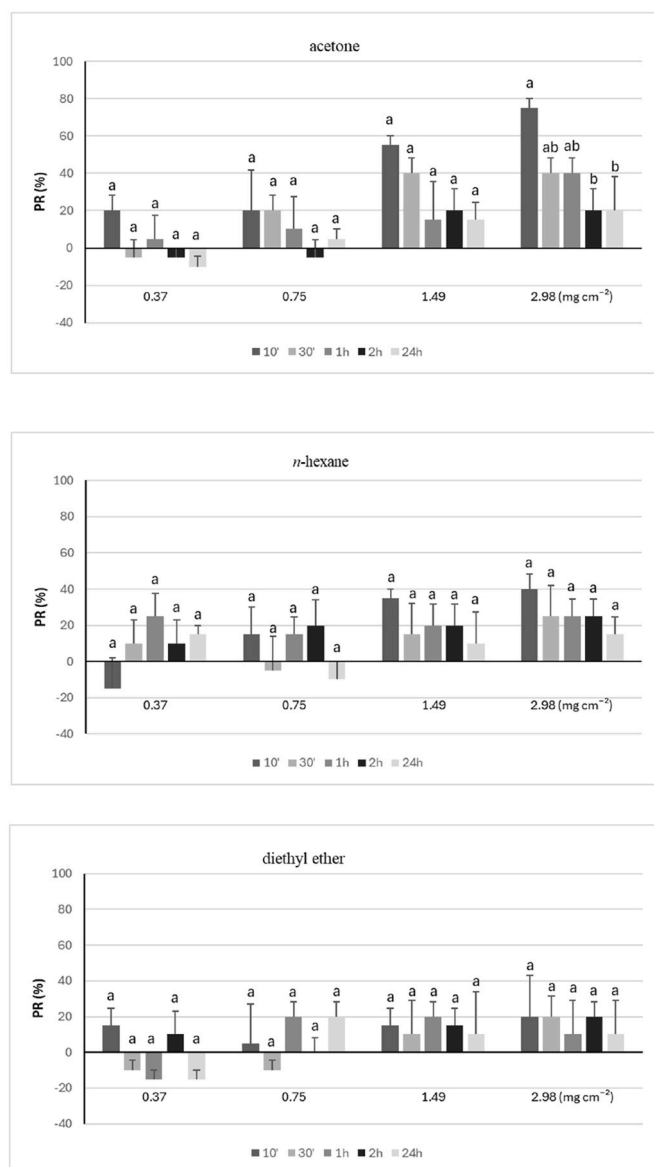


Fig. 3. Percent repellency (PR) (mean + S.E.) of different concentrations of pomegranate peel extracts against *E. kuehniella* larvae in filter paper disc bioassays. PR values were obtained from four independent replicates ($n = 40$ larvae per concentration) and reported as a function of dose and time after exposure. For each dose, different letters indicate significant differences among time at $p = 0.05$ (in all cases $df = 4, 10$; Tukey's test).

degradation, supporting their suitability for integrated pest management (Belgacem et al., 2021; Singh et al., 2023). Chemical characterization of the pomegranate peel acetone extract revealed hexadecanoic acid, 9,12-octadecadienoic acid, and sitosterols (isomeric mixture) as the predominant constituents. These compounds have been reported to exhibit biological activity against several insect species. For instance, 9, 12-octadecadienoic acid has previously been described as exhibiting pronounced insecticidal effects against whiteflies, mites, and *Aphis craccivora* Koch (Hemiptera: Aphididae) (Ling et al., 2022), while n-hexadecanoic acid has been toxic to the fourth larval instar of the dipterans *Aedes aegypti* (L.) (Culicidae), *Culex quinquefasciatus* Say (Culicidae), and *Anopheles stephensi* Liston (Culicidae) (Rahuman et al., 2000; Chandrasekaran et al., 2017). Such fatty acids may contribute to contact toxicity through nonspecific physicochemical effects, such as alterations in membrane integrity or facilitation of penetration of other bioactive constituents (Isman, 2020). Moreover, sitosterol has

Table 7

Repeated measures ANOVA for the repellent activity of acetone, n-hexane, and diethyl ether extracts of pomegranate fruit peel against *E. kuehniella* larvae in filter paper disc bioassays at four doses (2.98–0.37 mg/cm²) and five exposure times (10–120 min and 24 h).

Acetone extract			
Source	df	F	p
Between-subjects effects			
Dose	3, 12	4.926	0.019
Within-subjects effects			
Exposure time	4, 48	9.129	<0.001
Dose × Exposure time	12, 48	0.866	0.553
n-Hexane extract			
Source	df	F	p
Between-subjects effects			
Dose	3, 12	1.628	0.235
Within-subjects effects			
Exposure time	4, 48	0.949	0.444
Dose × Exposure time	12, 48	1.001	0.462
Diethyl ether extract			
Source	df	F	p
Between-subjects effects			
Dose	3, 12	1.172	0.361
Within-subjects effects			
Exposure time	4, 48	0.496	0.739
Dose × Exposure time	12, 48	0.756	0.691

¹ Degrees of freedom were corrected using the Greenhouse–Geisser adjustment when sphericity was violated.

demonstrated growth-regulatory and antifeedant activity against other lepidopteran species, such as the noctuids *Spodoptera littoralis* (Boisduval) and *Spodoptera litura* (F.), influencing growth rates and nutritional indices (Mukherjee, 2003; Santos et al., 2018). This could partially explain the strong feeding deterrence and negative nutritional parameters observed in the present study. It is important to underline that the biological response to plant-derived extracts can differ among insect taxa, even when using the same plant matrix and experimental design. Several studies have shown that plant extracts highly effective against one pest species may exhibit limited or no activity against others, reflecting interspecific differences in physiology, feeding behaviour, and detoxification capacity (Hussein et al., 2025; Arora et al., 2017; Kavallieratos et al., 2023, 2024; Saleem et al., 2019). Therefore, to further validate the potential use of pomegranate peel acetone extract, future studies are needed to assess its efficacy across multiple target species and to clarify the mechanisms of action of the active compounds. This would allow comparisons with synthetic insecticides acting through similar physiological pathways, as well as the assessment of their synergistic interactions and effects on non-target organisms. These insights could guide the development of stable, broad-spectrum pomegranate peel biopesticide formulations, contributing to the reduction of synthetic chemical inputs in insect post-harvest protection programs.

CRediT authorship contribution statement

Federica Lo Muzio: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Onofrio Marco Pistillo:** Formal analysis, Writing – original draft, Writing – review & editing. **Ilaria D'Isita:** Writing – review & editing. **Giovanni Iadarola:** Writing – review & editing. **Giuseppe Rotundo:** Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. **Antonella Di Palma:** Supervision, Writing – review & editing. **Antonio De Cristofaro:** Writing – review & editing. **Nickolas G. Kavallieratos:** Formal analysis, Supervision, Writing – review & editing. **Maria C. Boukouvala:** Formal analysis, Writing – review & editing. **Giacinto Salvatore Germinara:** Funding acquisition, Project administration, Supervision.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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7. Bioassay-guided fractionation of the active pomegranate peel extract towards two stored-product insect pests

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Bioassay-guided fractionation of the active pomegranate peel extract towards two stored-product insect pests

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1. Introduction

The stored-product insect pests *Sitophilus granarius* (L.) and *Ephestia kuehniella* (Zeller) are among the most economically important and cosmopolitan species affecting stored cereals (Buckland, 1981; Jacob & Cox, 1977; Panagiotakopulu et al., 2018; Güncan et al., 2023). Their persistence during long-term storage, along with their ability to exploit diverse food substrates and compromise market product value and food safety, makes their infestations one of the major problems in warehouses (Phillips & Throne, 2010; Pires et al., 2018). Historically, the control of these pests relied on chemical protectants, primarily pyrethroids and organophosphates, as well as fumigants (Arthur, 1996; Daglish et al., 2018; Kavallieratos et al., 2015). However, while contact insecticides are increasingly limited by resistance, environmental persistence, and human-health concerns, fumigants face similar constraints: methyl bromide was globally phased out in 2005, and phosphine is progressively losing effectiveness (Hamel et al., 2020; Nayak et al., 2020; Wakil et al., 2021; Machuca-Mesa et al., 2024). Therefore, alternative approaches aligned with integrated pest management (IPM) frameworks are increasingly being explored to control *S. granarius* and *E. kuehniella*. Several plant-derived products have shown promising activity against these pests. For instance, extracts from *Prunus serotina* (Ehrh.) leaves exhibit marked antifeedant effects on *S. granarius* adults (Sousa et al., 2025), while flower and leaf extracts of *Diplotaxis tenuifolia* (L.) demonstrate significant contact toxicity towards adults of this species (Tüfekçi, 2025). Conversely, strong insecticidal effects have been reported for *Ferulago angulata* (Schltdl.) and *Ferula*

assa-foetida (L.) essential oils against *E. kuehniella* larvae, causing clear dose-dependent mortality (Hamidian et al., 2024). Moreover, *Sideritis vulcanica* (Hub.-Mor.) essential oil has shown notable bioactivity on *E. kuehniella* larvae, reducing fecundity and affecting larval development (Keskin et al., 2025). In this regard, pomegranate (*Punica granatum* L.) peel represents another promising botanical matrix whose insecticidal activity has recently been demonstrated against *S. granarius* adults and *E. kuehniella* larvae (Lo Muzio et al., 2025). Chemical profiling of the acetone peel extract revealed multiple bioactive classes, including phytosterols, fatty acids, tocopherols, triterpenes, and phenolic–furanic derivatives, several of which are known to exert insecticidal effects (Aguilar-Marcelino et al., 2022; Lin et al., 2021; Younus et al., 2021; Daoui et al., 2022). Nevertheless, despite this chemical richness, the specific constituents responsible for the insecticidal activity of the acetone extract have not been conclusively identified. In light of these considerations (Building on these findings), the present study aimed to identify the active constituents responsible for the insecticidal activity previously observed. To this end, a bioassay-guided fractionation approach was applied to isolate the most toxic fraction and evaluate its biological effects on *S. granarius* adults and *E. kuehniella* larvae. By integrating chemical identification with comparative biological assays, this work provides novel mechanistic insights into the bioactivity of pomegranate peel extracts and contributes to the broader effort to develop effective plant-derived tools for stored-product protection.

2. Materials and methods

2.1 Insects

The insects used in the experiments were reared at the Department of Agriculture, Food, Natural Resources and Engineering, University of Foggia for several generations. Colonies of *E. kuehniella* was reared on a wheat-flour at 25 °C and 60 % RH in ventilated plastic containers. Second-instar larvae were obtained 7-9 days after hatching, which corresponds to the expected L2 developmental stage under these rearing conditions. Cohorts were synchronized by daily egg collection, and larvae were handled using a fine brush. When any uncertainty in larval staging occurred, instar identity was verified by measuring head capsule width according to Harvey and Thompson (1995) and Harvey and Vet (1997).

Adults of *Sitophilus granarius* were obtained from a laboratory colony maintained on whole wheat kernels at 26-27 °C, 65-70% RH, and continuous darkness. The culture had been reared for at least six generations under these controlled conditions and refreshed monthly with clean, uninfested wheat. Newly emerged adults were collected daily and held under the same environmental conditions. For all bioassays, adults of mixed sex, aged 2-4 weeks were used, as recommended in previous studies (Kljajić et al., 2014; Karas et al., 2017; Paventi et al., 2021).

2.2 Plant material and extraction

Pomegranate fruits (*Punica granatum* L., cv. Ako) were harvested at full ripeness in mid-September 2022 from organic orchards located in Foggia (Italy). Peels were manually removed, dried at 27 ± 2

°C for 15 days, ground, and sieved (0.5 mm mesh). Aliquots of the dried peel powder (100 g) were extracted for 24 h at room temperature with solvents of decreasing polarity: acetone, diethyl ether, and *n*-hexane (300 mL each). Each crude extract was centrifuged ($40,000 \times g$, 10 min, 10 °C), filtered (Whatman No. 113 paper), and concentrated under reduced pressure using a rotary evaporator (40

°C, 200 mbar). The resulting crude extracts were stored at -20 °C until further use.

2.3 Fractionation of extract by Silica Gel Column Chromatography

Based on the bioassays conducted by Lo Muzio et al. (2025), the acetone extract, which showed the highest biological activity against *S. granarius* adults and *E. kuehniella* second-instar larvae, was selected in the present study for bioactivity-guided fractionation and identification of its active constituents. An aliquot of the crude acetone extract (150 mg) was subjected to column chromatography on silica gel S (ϕ 1.5 cm $\times$ 20 cm), previously activated at 120 °C for 1 h. Elution was performed using a stepwise gradient of increasing acetone concentrations in *n*-hexane, consisting of the following solvent systems (v/v): 100:0, 80:20, 60:40, 40:60, and 20:80 (*n*-hexane:acetone), yielding 25 fractions (20 mL each), which were concentrated under a gentle nitrogen stream to a final volume of 1 mL. To ensure complete removal of residual *n*-hexane, 1.5 mL of acetone was added and evaporated again under nitrogen to obtain the same final volume. All fractions were subsequently evaluated for their contact toxicity against both insect species (see section 2.5).

2.4 TLC purification of the active column fraction

Preparative TLC was performed following standard procedures for non-UV-visible constituents (Wagner & Bladt, 1996; Stahl, 1969), with minor modifications. Fraction 22, identified as the most bioactive column fraction, was applied onto silica gel plates (5 × 20 cm, 0.25 mm; Merck) and developed with *n*-hexane:acetone (60:40, v/v). The solvent migration distance reached approximately 12 cm. Since the separated compounds were neither intrinsically colored nor UV-active, no discrete bands were detectable. To ensure full recovery of all potential constituents, the developed plate was therefore systematically divided into six consecutive 2-cm zones along the direction of solvent flow (from the origin to the solvent front). Each zone was scraped, and the collected silica was transferred into a Pasteur pipette plugged with a small cotton-wool pad acting as a filtration bed. Adsorbed material was eluted with acetone, and eluates were collected until reaching a final volume of 10 mL for each zone. The eluates were then concentrated under a gentle nitrogen stream to approximately 2 mL, yielding subfractions SF1-SF6.

An aliquot of each subfraction (50 µL) was diluted 1:50 for GC-MS analysis, whereas the remaining volume (1.95 mL) was further concentrated under nitrogen to 100 µL and used for topical bioassays (see section 2.5).

2.5 Bioassay of fractions

Contact toxicity bioassay was performed on *S. granarius* adults and *E. kuehniella* larvae using column fractions (Fr1-Fr25) or TLC subfractions (SF1-SF6), following the method described by Xie et al. (1996), with minor modifications. A 1 µL aliquot of each test solution was applied using a Hamilton microsyringe onto the pronotum of *S. granarius* adults in thanatosis (a natural temporary immobility response) and to the dorsal integument of *E. kuehniella* larvae under a stereomicroscope. Control insects received 1 µL of acetone. Both treatments and controls were conducted using three independent replicates of 5 insects each ($n = 15$ per group). Following application, *S. granarius* adults were individually transferred to Petri dishes containing five wheat kernels, whereas *E. kuehniella* larvae were placed in Petri dishes provided with 0.1 g of wheat flour. All assays were maintained in continuous darkness at $26\text{--}27 \pm 1$ °C, 65–70% relative humidity. Mortality was recorded 24, 48 and 72 h after treatment for column fractions whereas after 24h with TLC subfraction.

2.6 GC–MS analysis and quantification

Gas chromatography-mass spectrometry (GC-MS) analyses were performed using an Agilent 7890B gas chromatograph coupled to an Agilent 5977 mass selective detector (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separations were achieved on an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.5 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.25 mL min⁻¹. Samples (1 µL) were injected in splitless mode, with the injector temperature set at 250 °C. The oven temperature program was as follows: 100 °C (held for 5 min), increased to 280 °C at 6 °C min⁻¹, and held for 40 min. Mass spectra were acquired in electron impact (EI) ionization mode at 70 eV over an m/z range of 35–550, with a scan rate of 2.9 scans s⁻¹ and a solvent delay of 5 min. Compound identification was performed by comparison of mass spectra with the NIST11 library. Quantitative determinations were conducted using external calibration curves generated from analytical β-sitosterol standards (0.05–5.0 µg mL⁻¹). All analyses were carried out in triplicate.

2.7 Bioassays with commercial β-sitosterol

Commercial β-sitosterol (≥98% purity; Sigma-Aldrich) was tested as a reference compound to assess the biological activity of the sterol identified in the active subfraction on *S. granarius* adults and *E. kuehniella* larvae. Contact toxicity was evaluated using the same topical application procedure described in section 2.5. Mortality was recorded after 24, 48 and 72 hours after treatments. Ingestion toxicity was assessed with the flour-disk bioassay, and nutritional indices were calculated as reported in Lo Muzio et al. (2025). For both bioassays, stock solutions were prepared in acetone at four concentrations (600, 300, 150, and 75 µg mL⁻¹), and treatments were performed with three independent replicates of 10 insects each (n = 30 per treatment). Control insects received acetone only.

2.8 Data analysis

For statistical analysis, mortality percentages were arcsine square-root transformed and analyzed by one-way ANOVA. Data normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were verified before analysis, and significant differences among treatments were identified using Tukey's HSD test ($\alpha = 0.05$). Lethal doses (LD₅₀ and LD₉₀), along with their 95% confidence limits, regression equations, and chi-square (χ^2)

values, were estimated by probit analysis according to Finney (1971). For ingestion assays, variation in nutritional indices (RGR, RCR, ECI, FDI) across concentrations was assessed through one-way ANOVA, followed by Tukey's HSD test for post-hoc comparisons ($\alpha = 0.05$). All statistical analyses were performed using SPSS v.23 (IBM Corp., Armonk, NY, USA).

3. Results

3.1 Screening of column fractions for contact toxicity

Screening of the 25 chromatographic fractions for contact toxicity revealed Fraction 22 as the only fraction inducing measurable toxicity in both species. In particular, in adults of *S. granarius*, this fraction induced 80% of mortality at 24 h (ANOVA: $F_{(25,52)} = 21.26$, $p < 0.0001$) with no further increase at 48 and 72 h. In larvae of *E. kuehniella*, the same fraction induced a 66.7% of mortality at 24 h and 80% at 48 h (ANOVA 24 h: $F_{(25,52)} = 14.79$, $p < 0.0001$; 48 h: $F_{(25,52)} = 6.22$, $p < 0.0001$). In contrast, the remaining 24 fractions produced mortality levels not statistically different from the control, and none exceeded 13.3% mortality in either species at all observation times (24–72 h).

3.2 Screening of TLC subfractions

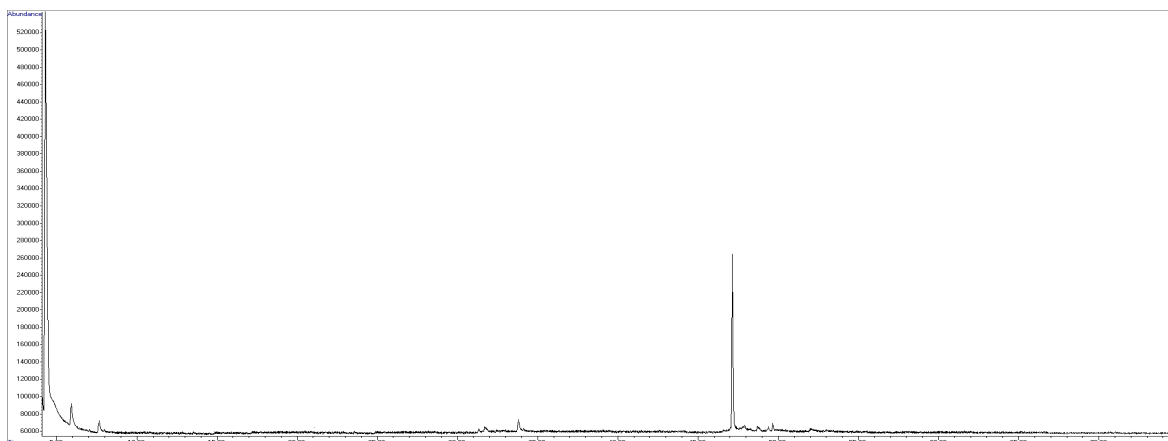
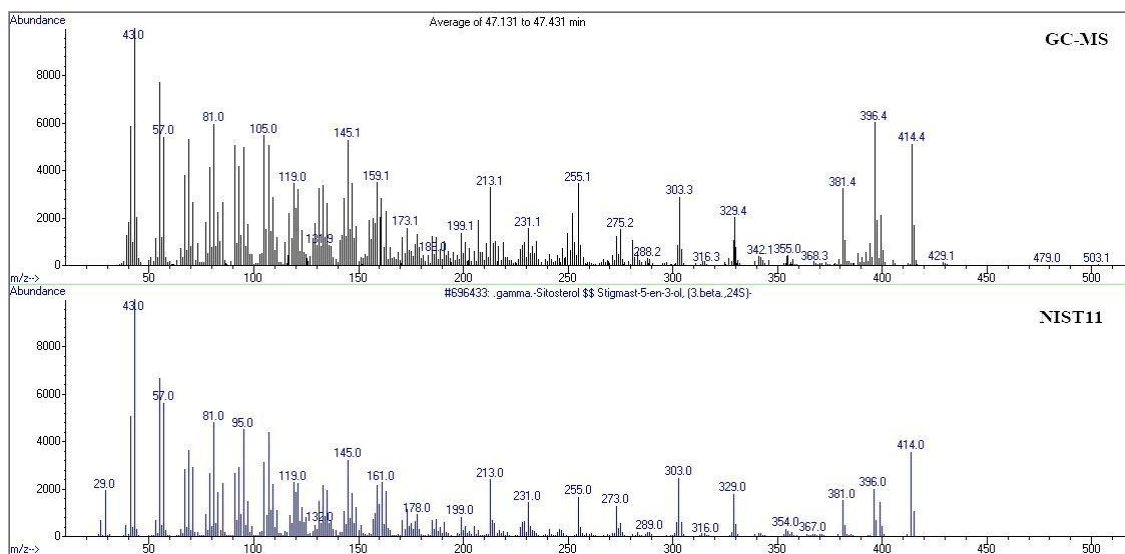
Contact toxicity bioassay conducted using the six TLC subfractions revealed a single active fraction in both insect species. After 24 h of exposure, subfraction 4 caused 70% mortality in *S. granarius* adults, with mortality differing significantly among treatments (ANOVA: $F_{(6,14)} = 107.75$, $p < 0.0001$). All other subfractions produced 0% mortality and did not differ statistically from the acetone control. A similar trend was observed in *E. kuehniella* larvae where Subfraction 4 caused 53.3% mortality and represented the only treatment significantly different from the control (ANOVA: $F_{(6,14)} = 125.83$, $p < 0.0001$).

3.3 Chemical characterization of the active subfraction

The GC-MS analysis of the bioactive TLC subfraction (SF4) revealed a chromatogram dominated by a single, well-defined peak. The EI mass spectrum showed the characteristic

fragmentation pattern of plant sterols, and the NIST library search identified the compound as belonging to the sitosterol class, returning a 92% match for β -sitosterol and a 98% match for γ -sitosterol.

To determine whether the isolated compound corresponded specifically to β -sitosterol, SF4 was analyzed in parallel with a commercial β -sitosterol standard ($\geq 98\%$ purity; Sigma-Aldrich) under identical chromatographic conditions. The two chromatographic profiles did not overlap: the retention time of SF4 differed markedly from that of the β -sitosterol standard, indicating that the compound present in SF4 is not β -sitosterol. Because γ -sitosterol is not readily available as an analytical standard (commercially limited and considerably more expensive), full stereochemical confirmation could not be achieved.



3.4 Contact toxicity of β -sitosterol

Contact toxicity of β -sitosterol towards *S. granarius* adults and *E. kuehniella* larvae at 24, 48 and 72 h are reported in Table 1 and 2. Across all exposure times, mortality increased with dose in both species. At the highest dose tested (600 μ g), *E. kuehniella* reached 38% mortality after 72 h, whereas

S. granarius reached a maximum of 20% under the same conditions. In contrast, the two lowest doses (150 and 75 μ g) caused zero mortality in *S. granarius* at any observation time, while only limited mortality was recorded in *E. kuehniella* ($\leq 16\%$). Probit analysis determined the LD₅₀ values for *E. kuehniella* larvae ranging from 989 to 1827 μ g/larvae, and from 1211 to 1468 μ g/adult for *S. granarius* adults.

Table 1. Contact toxicity of different concentrations of beta-sitosterol extract against *S. granarius* 24, 48 and 72 h after topical application.

Dose (μ g/adult)	Exposure time (h)	% Mortality (mean $\pm$ S.E.)	Regression equation	χ^2	LD50 (95% CL, μ g/adult)	LD90 (95% CL, μ g/adult)
600	24	16.00 $\pm$ 2.45 a	$y = -8.14 - 2.60x$	0.780	1392	4350
300		6.00 $\pm$ 2.45 b				
150		0.00 $\pm$ 0.00 c				
75		0.00 $\pm$ 0.00 c				
0		0.00 $\pm$ 0.00 c				
F		20.0				
d.f.		4				
p		< 0.001				
600	48	16.00 $\pm$ 2.45 a	$y = -7.50 - 2.37x$	1.51	1468	5105
300		8.00 $\pm$ 2.00 b				
150		0.00 $\pm$ 0.00 c				
75		0.00 $\pm$ 0.00 c				
0		0.00 $\pm$ 0.00 c				
F		25.6				
d.f.		4				
p		< 0.001				
600	72	20.00 $\pm$ 3.16 a	$y = -7.72 - 2.50x$	1.83	1211	3938
300		10.00 $\pm$ 3.16 b				
150		0.00 $\pm$ 0.00 c				
75		0.00 $\pm$ 0.00 c				
0		0.00 $\pm$ 0.00 c				
F		20.0				
d.f.		4				
p		< 0.001				

Note. For each exposure time, mean mortality values followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

Table 2. Contact toxicity of different concentrations of beta-sitosterol extract against *E. kuehniella* second instar larvae 24, 48 and 72 h after topical application.

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	χ^2	LD50 (95% CL, µg/adult)	LD90 (95% CL, µg/adult)
600	24	24.00 ± 5.10 a	$y = -4.42 - 1.36x$	0.418	1827	16087
300		16.00 ± 4.00 ab				
150		8.00 ± 3.74 bc				
75		2.00 ± 2.00 bc				
0		0.00 ± 0.00 c				
F		11.8				
d.f.		4				
P		< 0.001				
600	48	34.00 ± 6.00 a	$y = -3.81 - 1.26x$	1.26	1061	11067
300		30.00 ± 3.16 ab				
150		14.00 ± 11.40 bc				
75		6.00 ± 2.45 cd				
0		0.00 ± 0.00 d				
F		16.0				
d.f.		4				
P		< 0.001				
600	72	38.00 ± 5.83 a	$y = -3.40 - 1.13x$	0.63	989	13327
300		32.00 ± 3.74 ab				
150		16.00 ± 5.10 bc				
75		10.00 ± 3.16 c				
0		0.00 ± 0.00 d				
F		16.3				
d.f.		4				
P		< 0.001				

Note. For each exposure time, mean mortality values followed by the same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

3.5 Nutritional Effects of β -sitosterol on *S. granarius* and *E. kuehniella*

The ingestion toxicity, antifeedant response, and nutritional effects of β -sitosterol towards the target species are summarized in Tables 3 and 4. Overall, mortality remained low in both species, not exceeding 16% in *E. kuehniella*, while no mortality was recorded in *S. granarius* at any concentration.

In *E. kuehniella*, β -sitosterol produced a clear dose-dependent alteration of all nutritional parameters. Relative growth rate (RGR), relative consumption rate (RCR), and efficiency of conversion of ingested food (ECI) were significantly reduced at all concentrations compared with the control (RGR: $F_{4,20} = 11.26$, $p < 0.0001$; RCR: $F_{4,20} = 11.01$, $p < 0.0001$; ECI: $F_{4,20} = 9.71$, $p = 0.0002$). Feeding

deterrence similarly increased with dose, reaching values above 60% at 600 µg/disk (FDI: $F_{3,16} = 21.21$, $p < 0.0001$). In *S. granarius*, the RGR values did not differ significantly among treatments ($F_{4,20} = 2.42$, $p = 0.0825$), and the ECI indices showed only a marginal, non-significant reduction ($F_{4,20} = 2.84$, $p = 0.0514$). In contrast, β -sitosterol significantly reduced the relative consumption of food (RCR) in a dose-dependent manner ($F_{4,20} = 11.70$, $p < 0.0001$). Furthermore, feeding deterrence value reached a peak of 56.6% at the highest concentration tested (600 µg/disk).

Table 3. Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of beta-sitosterol in acetone solvent.

Concentration (µg/Disk)	Mortality (%)	RGR ¹	RCR	ECI	FDI (%)
600.00	0.00	-0.023 ± 0.016 a	0.167 ± 0.022 a	-10.847 ± 6.834 a	56.640 ± 6.779 a
300.00	0.00	-0.034 ± 0.013 a	0.261 ± 0.020 ab	-12.685 ± 4.551 a	29.360 ± 6.343 b
150.00	0.00	-0.033 ± 0.007 a	0.218 ± 0.016 b	-14.541 ± 2.547 a	31.750 ± 4.457 b
75.00	0.00	-0.008 ± 0.005 a	0.278 ± 0.022 bc	-2.943 ± 1.752 a	18.800 ± 6.679 b
Control	0.00	0.004 ± 0.008 a	0.343 ± 0.015 c	1.313 ± 2.119 a	

Note. Values followed by the same letter within each column are not significantly different at $p \leq 0.05$.

¹ RGR = relative growth rate; RCR = relative consumption rate; ECI = efficiency of conversion of ingested food; FDI = feeding deterrence index.

Table 4. Nutritional indices, mortality and food deterrence of *E. kuehniella* second instar larvae of different concentrations of beta-sitosterol in acetone solvent.

Concentration (µg/Disk)	Mortality (%)	RGR ¹	RCR	ECI	FDI (%)
600.00	12.00 ab	-0.023 ± 0.005 a	0.071 ± 0.011 a	-31.801 ± 4.349 a	64.377 ± 5.051 a
300.00	16.00 a	-0.017 ± 0.007 a	0.092 ± 0.009 ab	-19.498 ± 8.147 ab	57.847 ± 4.609 a
150.00	10.00 ab	-0.015 ± 0.009 a	0.109 ± 0.009 ab	-14.212 ± 8.584 ab	46.785 ± 5.892 a
75.00	4.00 ab	-0.001 ± 0.003 a	0.142 ± 0.005 bc	-1.005 ± 2.204 bc	15.347 ± 2.772 b
Control	0.00 b	0.021 ± 0.003 b	0.170 ± 0.022 b	15.456 ± 2.234 c	

Note. Values followed by the same letter within each column are not significantly different at $p \leq 0.05$.

¹ RGR = relative growth rate; RCR = relative consumption rate; ECI = efficiency of conversion of ingested food; FDI = feeding deterrence index.

4. Discussion

Over the past decades, botanically derived products have received increasing scientific attention as potential tools for the management of stored-product insects, driven by the demand for safer, residue-free, and environmentally compatible control strategies (Nikolaou et al., 2021; Murugesan et al., 2021; Ali et al., 2021; Akbar et al., 2022). A substantial body of literature indicates that pomegranate peel extracts can interfere with multiple physiological processes in both lepidopteran and coleopteran pests, exhibiting contact toxicity as well as antifeedant and growth-disrupting activity (Hamouda et al., 2015; El Namaky et al., 2020; Hamadani et al., 2021; Sakaan et al., 2024). However, the specific compounds responsible for these effects remain poorly defined.

Within this framework, the present study aimed to clarify the chemical and biological basis underlying the activity of the acetone pomegranate peel extract, previously identified as the most effective extract against *S. granarius* adults and *E. kuehniella* larvae (Lo Muzio et al., 2025). To achieve this objective, a bioassay-guided fractionation approach was used, which integrates sequential chromatographic separation with targeted biological screening to isolate constituents responsible for insecticidal activity. This strategy is widely recognized for its effectiveness in recovering bioactive molecules from botanical matrices (Torkey et al., 2009; De Feo et al., 2009; Khan et al., 2019; Fan et al., 2025). Among the 25 chromatographic fractions obtained, only one fraction (Fr22) induced substantial mortality (80% in *S. granarius* within 24 h and 66.7–80% in *E. kuehniella* within 24–48 h), whereas all other fractions remained not statistically different from the control. Purification of Fr22 further concentrated the activity into one subfraction (SF4), which caused 70% mortality in *S. granarius* and 53.3% in *E. kuehniella* after 24 h from exposure. The convergence of both bioassays toward the same fraction and subfraction provides strong evidence for the presence of a dominant, well-resolved bioactive constituent, a pattern frequently observed in bioassay-guided isolations of insecticidal plant metabolites (Rotundo et al., 2019; Abutaha et al., 2020; Ali et al., 2024; Fan et al., 2025).

However, the two species, did not respond identically to the active fraction and subfraction, with *S. granarius* adults exhibited faster and higher mortality than *E. kuehniella* larvae. Similar interspecific differences have been reported for structurally related sterols, which often show distinct toxicity profiles across insect species. For instance, stigmasterol was highly toxic to *Rhyzopertha dominica* but resulted inactive

against *Tribolium castaneum* (Díaz et al., 2015), while cholestanone induced greater mortality in *Mythimna separata* than in *Spodoptera frugiperda* (Tang et al., 2022). Moreover, Renoz et al. (2021) demonstrated that insect sterol metabolism is species-specific, and certain sterols become toxic only when key metabolic pathways are absent. This selectivity reflects differences in sterol assimilation, transformation, and excretion among insect taxa (Svoboda & Lusby, 1994; Boukid et al., 2021; Papanikolaou et al., 2021; Janson et al., 2009; Svoboda & Thompson, 2019; Svoboda, 2020).

Chemical characterization of SF4 revealed the presence of a dominant sterol belonging to the sitosterol family, as indicated by its EI mass fragmentation pattern and high similarity scores returned by the NIST library for β -sitosterol (92%) and γ -sitosterol (98%). This assignment is consistent with the absence of ultraviolet-absorbing chromophores (i.e., the absence of visible bands on the TLC plate at 254 or 366 nm), a characteristic profile of non-conjugated mid-polar metabolites like phytosterols (Liu & Ruan, 2013; Nzekoue et al., 2020). To preserve sample integrity for subsequent biological testing, visualization reagents were not employed (Liu & Ruan, 2013; Nzekoue et al., 2020).

To determine whether the compound corresponded specifically to β -sitosterol, the sterol peak isolated from SF4 was analysed alongside a commercial β -sitosterol standard under identical GC–MS conditions. The retention time of the SF4 sterol differed markedly from that of the reference, indicating the presence of a structural isomer. Since γ -sitosterol standards are rarely available and often prohibitively expensive, β -sitosterol was used as a structurally representative molecule to evaluate whether the sitosterol backbone could contribute to the biological activity observed in the purified fraction.

The phytochemical literature on *P. granatum* peel also points to a complex sterol profile. Several studies report β -sitosterol as a major component (Bayati et al., 2020; Nweze et al., 2019; Sun et al., 2021), whereas others also detect γ -sitosterol (Yassin et al., 2021; Hussain et al., 2024). Similar discrepancies are common in sterol reports from other botanicals, where isomer identity and relative abundance vary across studies. Whether these differences rely on genotype, environmental and agronomic conditions, fruit developmental stage, and solvent-dependent extraction selectivity, is a matter of speculation (Münger et al., 2018; Booter et al., 2023; Valitova et al., 2016).

The toxicological profiling of commercial β -sitosterol induced only limited contact mortality (20% in *S. granarius* and 38% in *E. kuehniella* at the maximum tested dose of 600 μ g). More importantly, in ingestion assays, this compound did not elicit strong

lethality ($\leq 16\%$ in *E. kuehniella*, 0% in *S. granarius*), however, it substantially disrupted nutritional physiology. In *E. kuehniella* larvae, all nutritional indices (RGR, RCR, ECI) were significantly impaired across doses, and feeding deterrence increased in a dose-dependent manner, exceeding 60% at the highest concentration. In *S. granarius*, β -sitosterol significantly reduced RCR and produced an FDI of 56.6% at 600 $\mu\text{g}/\text{disk}$, indicating strong antifeedant activity.

These findings are in line with previous evidence showing that β -sitosterol generally acts as a metabolic or growth-modifying agent. For instance, dietary β -sitosterol significantly reduces larval growth and adult emergence in *Helicoverpa armigera* but induces limited lethality (Mishra et al., 2020). Moreover, in *Henosepilachna vigintioctopunctata*, β -sitosterol isolated from *Andrographis paniculata* (L.) produced a clear antifeedant response while only limited larvicidal and adulticidal effects (Revathi, 2021). In *Spodoptera littoralis*, dos Santos et al. (2019) demonstrated that a sterol-rich fraction containing β -sitosterol, stigmasterol and campesterol significantly reduced food intake, with growth inhibition entirely explained by decreased ingestion.

In contrast, γ -sitosterol has been shown to possess intrinsic insecticidal activity. Nyamoita (2013) isolated γ -sitosterol as one of four individual compounds from *Vitex schiliebenii* and demonstrated that it induced substantial larval mortality in *Anopheles gambiae*, 35%, 45%, and 65% at 1, 5, and 10 ppm, respectively, alongside clear growth-regulatory effects. Verma et al. (2023) identified γ -sitosterol (22.1%) in the oleoresin of *Globba sessiliflora*, a preparation exhibiting pronounced insecticidal activity against *Lipaphis erysimi* and *Selepa celtis*, as well as strong nematotoxic effects on *Meloidogyne incognita*.

Overall, this study shows that the insecticidal properties of pomegranate peel acetone extract can be progressively narrowed from a crude extract to a single active chromatographic subfraction and attributed to a dominant sterol isomer. While the stereochemical identity of the active compound could not be fully resolved, the GC-MS evidence strongly supports a γ -sitosterol-type structure, and its precise elucidation will require dedicated standards or NMR-based analyses. A further limitation is the lack of formulation and stability assessments, which will be essential to determine the performance of the active molecule under realistic storage conditions. From an applied perspective, phytosterols offer a biodegradable, eco-friendly substitute to synthetic pesticides, reducing the risk of resistance in stored product pests (Tran et al., 2024; Muthu et al., 2025).

The identification of a sterol-like compound with rapid topical toxicity and marked species specificity opens a new line of investigation into the ecological role of plant sterols and their potential exploitation in stored-product protection. Rather than offering a finished product, these findings establish the chemical and biological framework upon which future formulation, delivery, and mode-of-action studies can be built, ultimately advancing the integration of plant-derived sterols into sustainable pest management strategies.

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8. Evaluation of globe artichoke (*Cynara cardunculus* L.) outer bract extracts against *Sitophilus granarius* (L.)

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Abstract

The industrial processing of globe artichoke (*Cynara cardunculus* L.) generates large amounts of outer bracts, a lignified by-product whose potential in pest management remains largely unexplored. In the present study, crude extracts obtained from globe artichoke outer bracts using acetone, *n*-hexane, and diethyl ether were evaluated against the granary weevil, *Sitophilus granarius* (L.), through contact and ingestion bioassays. Contact toxicity was strongly dependent on the extraction solvent, with the acetone extract showing the highest efficacy. At 150 µg/adult, the acetone fraction caused 36.67% mortality after 24 h and 50.00% after 48 h, with LD₅₀ values decreasing from 268.96 to 172.86 µg/adult over time. The *n*-hexane extract showed lower toxicity, whereas the diethyl ether extract was largely ineffective. In contrast, ingestion assays revealed negligible effects on mortality, feeding activity, and nutritional parameters, with no significant differences detected in relative growth rate, relative consumption rate, efficiency of conversion of ingested food, or feeding deterrence index. Overall, these results suggest that the bioactive constituents present in artichoke outer bracts exert their activity primarily through contact exposure. The findings provide the first evidence that globe artichoke outer bracts represent a biologically active agro-industrial residue with potential for valorization in the development of sustainable control mean for stored-product pest management.

Keywords: artichoke, bracts, industrial waste, by-products, granary weevil

1. Introduction

Industrial processing of globe artichoke (*Cynara cardunculus* L. var. *scolymus*) generates substantial quantities of solid residues, including stems, leaves, and outer bracts, which may account for more than 60% of the harvested material depending on cultivar and processing conditions (Noriega-Rodríguez et al., 2020; Francavilla et al., 2021; Laghezza Masci et al., 2024). Outer bracts represent a major fraction of the processing waste stream due to their structural characteristics, including high fiber content and tissue lignification, which limit their suitability for human consumption (Melini et al., 2025).

Despite being discarded, these tissues are not chemically inert and have been reported to contain high levels of phenolic compounds, particularly hydroxycinnamic acids such as chlorogenic acid and dicaffeoylquinic derivatives, along with flavonoids and other secondary metabolites (Jiménez-Moreno et al., 2019; Bavaro et al., 2025; Alves-Silva et al., 2024). Lipophilic constituents, including sterols and fatty acids, have also been identified (Dabbou et al., 2017; Feiden et al., 2023), further contributing to the chemical heterogeneity of these matrices.

Accordingly, artichoke outer bracts are increasingly considered a valuable resource, supported by evidence of their biological activity. In particular, they have been found to exhibit antioxidant activity and to show an inhibitory effect against *Aspergillus parasiticus* (Kollia et al., 2017).

More recently, artichoke residues, including bracts, have been explored in applied contexts. Their use as a functional filler in biodegradable poly(butylene succinate-co-adipate) (PBSA) composites has been associated with antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, along with improved biodegradability in soil environments (Minichiello et al., 2026), while hydrolyzed artichoke agro-industrial residues have also been used as fermentation substrates by *Komagataeibacter rhaeticus* QK23 for bacterial cellulose production, yielding structurally functional biopolymers (Quiñones-Cerna et al., 2025).

In contrast, their potential use in pest management remains poorly explored. Evidence of insecticidal activity has been reported for *C. cardunculus*, as leaf extracts were found to induce mortality in *Phenacoccus solenopsis*, with enhanced efficacy when combined with Zamzam water (Abd-Allah, 2022). Nevertheless, these observations are limited to specific plant tissues and insect species and cannot be directly extended to other fractions such as outer bracts.

The granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), is one of the most destructive pests of stored cereals, causing severe quantitative and qualitative losses and presenting significant challenges for control due to its concealed development within grain kernels (Diab et al., 2025; Zhong et al., 2026).

To date, studies specifically addressing the insecticidal potential of artichoke outer bracts are lacking. The insecticidal activity of artichoke bract extracts was therefore evaluated against *S. granarius* to assess their use in the control of stored-product pests.

2. Materials and Methods

2.1 Insects

The granary weevil, *S. granarius* (L.), was maintained as a laboratory population at the Department of Agriculture, Food, Natural Resources and Engineering (University of Foggia). The colony originated from adults collected in local grain storage facilities in 2021 and has since been continuously reared at 25 ± 1 °C, $65 \pm 5\%$ relative humidity, and in complete darkness. Insects were kept on whole wheat kernels (*Triticum aestivum* L.) placed in glass containers (15 × 15 cm) covered with a fine metallic mesh to ensure ventilation. For the bioassays, unsexed adults of known age were obtained by daily checking adult emergence from the stock culture. Newly emerged individuals were collected every 24 h, transferred to fresh wheat kernels, and used as a synchronized cohort in the experiments.

2.2 Plant Material

Outer bracts of globe artichoke (cv. Madrigal) were collected in May 2023 from an organically managed commercial in the Foggia rural area (southern Italy), belonging to the FIMAGRI cooperative. The plant material was dried at 35 ± 2 °C for 10 days under forced-air conditions to preserve its chemical integrity, then ground and sieved (0.5 mm mesh; ASTM E11 No. 35; Fritsch GmbH, Idar-Oberstein, Germany). For extraction, 100 g of powdered material were mixed with 300 mL of acetone, diethyl ether, or n-hexane and kept in the dark at room temperature (25 °C) for 24 h, with intermittent manual agitation. The mixtures were centrifuged (40,000 rpm, 10 min, 10 °C), and the supernatant was filtered through Whatman No. 113 filter paper (Cytiva, Marlborough, MA, USA). Solvents were removed under reduced pressure using a rotary evaporator (40 °C, 200 mbar; Laborota 4000, Heidolph, Schwabach, Germany). The obtained extracts were stored at -20 °C until

use.

2.3 Contact toxicity bioassay

The contact toxicity of artichoke bract extracts against *S. granarius* adults was evaluated by topical application. Extracts were dissolved in their corresponding solvents (acetone, diethyl ether, and n-hexane), and a series of two-fold dilutions was prepared, ranging from 150.00 to 18.75 µg/µL. A volume of 0.5 µL of each dilution was applied directly to the pronotum of each insect using a 700 series Microliter™ syringe (Hamilton Company, Reno, NV, USA). Prior to treatment, adults were immobilized by exploiting their natural thanatosis behaviour. For each concentration, three replicates of 10 unsexed adults were tested. Control groups consisted of insects treated with the corresponding solvent only. After treatment, insects were placed in Petri dishes within a cylindrical metal arena (4.0 cm diameter × 2.5 cm height), covered with a metallic mesh (1 mm) to prevent escape. Five wheat kernels were provided as a food source. All experimental units were maintained in darkness at 26 ± 2 °C and $60 \pm 5\%$ relative humidity. Mortality was assessed at 24 and 48 h after treatment. Individuals were considered dead when no movement was observed following gentle stimulation with a fine camel hairbrush. Mortality data were arcsine square-root transformed prior to analysis and subjected to one-way ANOVA, followed by Tukey's HSD test for mean separation. Assumptions of normality and homogeneity of variances were verified using Shapiro–Wilk and Levene's tests, respectively. Lethal doses (LD₅₀ and LD₉₀), along with confidence intervals, regression parameters, and chi-square (χ^2) values, were estimated by probit analysis (Finney, 1971). All statistical analyses were performed using SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA).

2.4 Feeding activity and nutritional indices

The effects of artichoke bract extracts on feeding behavior and nutritional indices of adult *S. granarius* were assessed using a flour disk bioassay based on the method described by Xie (1996). A suspension was prepared by mixing 2.5 g of wheat flour with 10 mL of distilled water under continuous magnetic stirring until a homogeneous mixture was obtained. Aliquots of 100 µL were then dispensed onto plastic Petri dishes to form flour disks, which were left to dry overnight under controlled conditions (26 ± 2 °C, $60 \pm 5\%$ RH) to ensure uniform consistency. Each disk was treated with 5 µL of extract solution to

obtain final doses of 93.75, 187.50, 375.00, and 750.00 µg per disk. Control disks received the corresponding solvent only. After treatment, all disks were left at room temperature for 2 h to allow complete solvent evaporation before insect exposure. For each replicate, two treated disks were placed in a pre-weighed glass vial (2.5 cm diameter × 4.0 cm height), and their initial weight was recorded. Subsequently, groups of ten adults (previously weighed together) were introduced into each vial. The vials were then re-weighed and maintained under controlled conditions (26 ± 2 °C, $60 \pm 5\%$ RH, darkness) for 72 h. At the end of the exposure period, mortality was recorded, and both the remaining flour disks and surviving insects were weighed separately. For each concentration and control, five replicates (10 insects per replicate) were performed simultaneously.

The following nutritional indices were calculated for each replicate:

$$\text{Relative Growth Rate (RGR)} = (A - B) / B \text{ day}^{-1}$$

$$\text{Relative Consumption Rate (RCR)} = D / B \text{ day}^{-1}$$

$$\text{Efficiency of Conversion of Ingested food (ECI)} = (RGR / RCR) \times 100$$

$$\text{Feeding Deterrence Index (FDI)} = (C - T) / C \times 100$$

A represents the mean weight (mg) of surviving insects at the end of the experiment (day 3), whereas

B corresponds to their initial mean weight (mg). C indicates the amount of food consumed in the control treatment, while T refers to the consumption recorded in treated disks. D represents the ingested biomass (mg), expressed per surviving insect at the end of the exposure period. Prior statistical analysis, data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene’s test. Differences among treatments were then analyzed by one-way ANOVA, followed by Tukey’s HSD test for mean comparison.

3. Results

3.1 Contact toxicity

Topical application of artichoke outer bract extracts induced different levels of mortality in *S. granarius* adults depending on solvent type, dose, and exposure time (Tables 1-3). Among the tested extracts, the acetone fraction showed the highest activity. At 24 h, mortality reached $36.67 \pm 6.67\%$ at 150 µg/adult, whereas at 48 h mortality increased to $50.00 \pm 11.55\%$ at the same dose. Significant dose-dependent differences were detected

for acetone at both exposure times (24 h: $F = 16.10$, $p = 0.0002$; 48 h: $F = 14.46$, $p = 0.0004$). Probit analysis confirmed the higher efficacy of this extract, with LD_{50} values decreasing from 268.96 $\mu\text{g}/\text{adult}$ at 24 h to 172.86 $\mu\text{g}/\text{adult}$ at 48 h.

The *n*-hexane extract showed lower activity overall. At 24 h, mortality ranged from 0 to $13.33 \pm 6.67\%$, and differences among concentrations were not significant ($F = 3.20$, $p = 0.0618$). After 48 h, mortality slightly increased, reaching $16.67 \pm 8.82\%$ at 150 $\mu\text{g}/\text{adult}$, and the treatment effect became significant ($F = 5.24$, $p = 0.0154$). However, LD_{50} and LD_{90} values remained markedly higher than those estimated for acetone, indicating lower toxicity.

The diethyl ether extract was the least effective. At 24 h, only the highest concentration caused measurable mortality ($13.33 \pm 6.67\%$), although the overall treatment effect was significant ($F = 4.00$, $p = 0.0343$). At 48 h, mortality increased to $20.00 \pm 5.77\%$ at 150 $\mu\text{g}/\text{adult}$, while intermediate doses produced $6.67 \pm 3.33\%$ mortality. Nevertheless, probit analysis did not provide reliable LD_{50} and LD_{90} estimates for this extract.

Table 1. Contact toxicity of different concentrations of *n*-hexane artichoke extract against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

Dose ($\mu\text{g}/\text{adult}$)	Exposure time (h)	% Mortality (mean $\pm$ S.E.)	Regression equation	χ^2	LD_{50} (95% CL, $\mu\text{g}/\text{adult}$)	LD_{90} (95% CL, $\mu\text{g}/\text{adult}$)
150	24	13.33 ± 6.67 a	$y = -5.986 + 2.275x$	0.770	430.43 (205.88–4.7 $\times$ 103)	1576.88 (423.86–6.9 $\times$ 103)
75		6.67 ± 3.33 a				
37.5		0.00 ± 0.00 a				
18.75		0.00 ± 0.00 a				
0		0.00 ± 0.00 a				
F		3.20				
d.f.		4				
p		0.0618				
150	48	16.67 ± 8.82 a	$y = -3.407 + 1.148x$	2.035	930.87 (248.06–2.9 $\times$ 105)	12179.16 (978.60–1.4 $\times$ 106)
75		10.00 ± 0.00 a				
37.5		10.00 ± 0.00 a				
18.75		0.00 ± 0.00 b				
0		0.00 ± 0.00 b				
F		5.24				
d.f.		4				
p		0.0154				

Table 2. Contact toxicity of different concentrations of acetone artichoke extract against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	χ^2	LD50 (95% CL, µg/adult)	LD90 (95% CL, µg/adult)
150	24	36.67 ± 6.67 a	$y = -3.622 + 1.491x$	0.689	268.96 (145.47–2343.17)	1947.39 (532.16–332750.34)
75		16.67 ± 3.33 ab				
37.5		13.33 ± 3.33 b				
18.75		3.33 ± 3.33 c				
0		0.00 ± 0.00 c				
F		16.10				
d.f.		4				
p		0.0002				
150	48	50.00 ± 11.55 a	$y = -3.836 + 1.714x$	3.330	172.86 (112.03–500.69)	966.56 (380.04–15056.98)
75		20.00 ± 5.77 ab				
37.5		16.67 ± 3.33 b				
18.75		3.33 ± 3.33 c				
0		0.00 ± 0.00 c				
F		14.46				
d.f.		4				
p		0.0004				

Table 3. Contact toxicity of different concentrations of dietyl ether artichoke extract against *S. granarius* adults 24 and 48 h after topical application. For each exposure time, mean mortality values followed by same letter are not significantly different at $p \leq 0.05$ (Tukey HSD test).

Dose (µg/adult)	Exposure time (h)	% Mortality (mean ± S.E.)	Regression equation	χ^2	LD50 (95% CL, µg/adult)	LD90 (95% CL, µg/adult)
150	24	13.33 ± 6.67 a	$y = -11.365 + 4.708x$	0.171	—	—
75		0.00 ± 0.00 a				
37.5		0.00 ± 0.00 a				
18.75		0.00 ± 0.00 a				
0		0.00 ± 0.00 a				
F		4.00				
d.f.		4				
p		0.0343				
150	48	20.00 ± 5.77 a	$y = -2.637 + 0.762x$	1.208	—	—
75		6.67 ± 3.33 ab				
37.5		6.67 ± 3.33 ab				
18.75		6.67 ± 3.33 ab				
0		0.00 ± 0.00 b				
F		3.24				
d.f.		4				
p		0.0601				

3.2 Feeding activity and nutritional indices

he ingestion bioassay revealed limited effects of artichoke bract extracts on *S. granarius* adults (Table 4-6). Mortality remained negligible across all tested concentrations and solvent extracts, with values not exceeding 4% even at the highest dose. No significant differences were observed in the nutritional indices (RGR, RCR, and ECI) among treatments for any of the tested extracts (one-way ANOVA, $p > 0.05$). The values recorded for treated groups were comparable to those of the control, suggesting that food consumption and nutrient conversion were not substantially affected by the ingestion of treated flour disks. Similarly, the feeding deterrence index (FDI) showed no statistically significant differences among treatments. **Table 4.** Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations acetone artichoke outer bracts extract. Means in the same column with the same letter are not significant different at the 0.05 level determined by the Tukey's HSD test.

Table 4 .Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of *n*-hexane extract. Means in the same column with the same letter are not significantly different at the 0.05 level determined by the Tukey's HSD test.

Concentration (µg/Disk)	Mortality (%)	RGR ¹	RCR	ECI	FDI (%)
750.00	0.00 a	0.017 ± 0.005 a	0.162 ± 0.007 a	10.613 ± 3.322 a	6.857 ± 3.674 a
375.00	0.00 a	0.012 ± 0.007 a	0.167 ± 0.015 a	6.752 ± 4.658 a	6.857 ± 6.595 a
187.50	0.00 a	0.021 ± 0.000 a	0.179 ± 0.009 a	12.043 ± 1.117 a	1.048 ± 6.595 a
93.75	0.00 a	0.020 ± 0.000 a	0.185 ± 0.009 a	10.532 ± 2.130 a	-1.000 ± 4.132 a
Control	0.00 a	0.023 ± 0.000 a	0.267 ± 0.010 a	13.058 ± 1.630 a	

¹ RGR, relative growth rate; RCR, relative consumption rate; ECI, efficiency conversion of ingested food; FDI, feeding deterrent index.

Table 5 .Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of acetone extract. Means in the same column with the same letter are not significantly different at the 0.05 level determined by the Tukey's HSD test.

Concentration (µg/Disk)	Mortality (%)	RGR ¹	RCR	ECI	FDI (%)
750.00	0.00 a	0.020 ± 0.002 a	0.167 ± 0.015 a	11.729 ± 1.273 a	15.500 ± 3.304 a
375.00	0.00 a	0.014 ± 0.002 a	0.148 ± 0.015 a	9.701 ± 1.273 a	24.125 ± 2.619 a
187.50	0.00 a	0.020 ± 0.002 a	0.157 ± 0.015 a	12.893 ± 1.273 a	22.750 ± 3.830 a
93.75	0.00 a	0.018 ± 0.002 a	0.170 ± 0.015 a	10.890 ± 1.273 a	15.500 ± 5.980 a
Control	0.00 a	0.020 ± 0.002 a	0.185 ± 0.015 a	11.392 ± 1.273 a	

¹ RGR, relative growth rate; RCR, relative consumption rate; ECI, efficiency conversion of ingested food; FDI, feeding deterrent index.

Table 6 .Nutritional indices, mortality and food deterrence of *S. granarius* adults of different concentrations of diethyl ether extract. Means in the same column with the same letter are not significantly different at the 0.05 level determined by the Tukey's HSD test.

Concentration ($\mu\text{g/Disk}$)	Mortality (%)	RGR ¹	RCR	ECI	FDI (%)
750.00	4.00 a	0.011 ± 0.007 a	0.150 ± 0.018 a	7.500 ± 5.178 a	19.145 ± 10.145 a
375.00	4.00 a	0.022 ± 0.010 a	0.172 ± 0.018 a	10.967 ± 4.898 a	13.115 ± 11.001 a
187.50	0.00 a	0.011 ± 0.007 a	0.142 ± 0.006 a	7.752 ± 5.162 a	12.770 ± 3.613 a
93.75	0.00 a	0.025 ± 0.009 a	0.171 ± 0.017 a	13.906 ± 4.240 a	1.385 ± 10.712 a
Control	0.00 a	0.040 ± 0.009 a	0.211 ± 0.023 a	18.954 ± 2.170 a	

¹ RGR, relative growth rate; RCR, relative consumption rate; ECI, efficiency conversion of ingested food; FDI, feeding deterrent index.

4. Discussion

Globe artichoke by-products have been extensively characterized in terms of their chemical composition and associated biological properties, primarily with respect to antioxidant and antimicrobial activities, whereas their potential role in insect control remains comparatively underexplored. Within this framework, the present findings demonstrate that outer bracts of *Cynara cardunculus* retain measurable insecticidal activity against *S. granarius* adults following topical exposure, with efficacy strongly modulated by the extraction solvent. Among the tested extracts, the acetone fraction exhibited the highest activity ($\text{LD}_{50} = 172.86 \mu\text{g/adult}$ at 48 h), whereas the n-hexane extract showed reduced toxicity and the diethyl ether extract was largely ineffective, indicating that compounds associated with contact toxicity are preferentially recovered in fractions of intermediate polarity.

Direct comparisons with the literature remain inherently limited, as studies specifically addressing crude extracts obtained from globe artichoke outer bracts are currently lacking. Moreover, relatively few investigations have examined contact toxicity of Asteraceae-derived products against *S. granarius*, since most available data concern fumigant activity of essential oils. Within this limited framework, Kordali et al. (2006) reported that essential oils from *Artemisia absinthium* and related species induced mortality rates of approximately 80–90% in *S. granarius* adults after 48 h of exposure at $9 \mu\text{L/L}$ air, with individual components such as 1,8-cineole and terpinen-4-ol achieving complete mortality at all tested doses. The higher efficacy reported for essential oils reflects their distinct chemical nature: these products are enriched in low-molecular-weight terpenoids with

rapid neurotoxic and fumigant activity, acting principally through interference with the octopaminergic nervous system of insects (Enan, 2001; Regnault-Roger et al., 2012). By contrast, the present work focused on crude solvent extracts obtained from a lignified and structurally specialized plant fraction, in which both the concentration and accessibility of highly active constituents are expected to be comparatively reduced. Further insight is provided by evidence within the same plant species. Abd-Allah (2022) showed that leaf extracts of *C. cardunculus* exert significant insecticidal activity against *Phenacoccus solenopsis*, with mortality reaching 70% with tap water-based preparations and increasing to 86.67% when formulated with Zamzam water at a concentration of 15,000 ppm. Although not directly comparable in terms of target species and experimental design, these findings confirm that *Cynara* tissues contain bioactive constituents capable of affecting insect survival and emphasize the role of formulation and solvent matrix in modulating biological efficacy. Accordingly, the moderate activity observed for outer bracts is consistent with their structural and metabolic characteristics, suggesting that this lignified by-product represents a chemically less optimized, yet still functionally relevant, reservoir of bioactive compounds.

More generally, the influence of plant organ on insecticidal performance is well documented across different botanical systems and reflects the heterogeneous distribution of secondary metabolites within plant tissues. For instance, Sarma et al. (2019) reported differential insecticidal activity between leaf and peel essential oils of *Citrus aurantifolia* against *Aedes aegypti* larvae and eggs, with the two fractions differing both in chemical composition and in the type of biological activity expressed. Similarly, studies on other species have consistently shown that metabolically active tissues such as leaves outperform structural tissues such as bark or stem in terms of bioactive compound content and associated pesticidal efficacy (Dolma et al., 2022; Zerihun et al., 2018). Collectively, these observations indicate that insecticidal efficacy is strongly dependent on the specific plant organ used for extraction, reflecting differences in metabolite concentration, accessibility, and chemical diversity. This interpretation is consistent with the documented chemical heterogeneity of *C. cardunculus*. Previous studies have shown that phenolic compounds are unevenly distributed among its organs, with the receptacle generally richer in hydroxycinnamic acids and leaves relatively richer in flavonoids than the outer bracts (Pandino et al., 2011; Ramos et al., 2014). In this context, the lower activity observed for outer bracts may reflect differences in the composition and extractability of bioactive constituents associated with this lignified tissue.

A second major outcome of the present study is the clear divergence between contact and ingestion bioassays. While topical application resulted in measurable mortality, ingestion did not significantly affect survival, feeding behaviour, or nutritional indices. Such route-dependent responses are widely reported for plant-derived insecticides and are generally attributed to differences in compound bioavailability, absorption dynamics, and metabolic detoxification following oral uptake (Isman, 2020). In the present case, the absence of oral activity suggests that the active constituents present in artichoke bracts are either insufficiently released within the flour- disk matrix or subject to rapid biotransformation in the digestive tract of *S. granarius*, rendering them inactive before reaching systemic targets.

Overall, the present study provides, to the best of our knowledge, the first evidence that crude extracts derived from globe artichoke outer bracts can affect *S. granarius* through contact exposure. Although their potency is lower than that reported for essential oils or extracts obtained from metabolically active tissues, the observed activity remains biologically meaningful in view of the nature of the tested material. The relevance of this matrix lies in its abundance, low cost, and high valorization potential as an agro-industrial by-product. Future research should focus on identifying the compounds responsible for the contact activity of the acetone fraction, elucidating possible synergistic interactions among constituents, and evaluating the efficacy of these extracts under realistic grain storage conditions.

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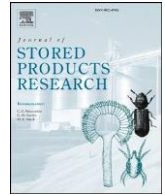
9. Optimizing λ -cyhalothrin for the effective control of *Alphitobius diaperinus*: Temperature and food effect revealed

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Optimizing λ -cyhalothrin for the effective control of *Alphitobius diaperinus*: Temperature and food effect revealed

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ABSTRACT

The lesser mealworm, *Alphitobius diaperinus*, presents substantial challenges as a secondary pest in global stored-product management, especially within the poultry industry. This study examines the effectiveness of λ -cyhalothrin, a synthetic pyrethroid, against *A. diaperinus* larvae and adults under various abiotic and biotic conditions. Bioassays were conducted at both half-label and label doses across different post-exposure temperatures ranging from 20 to 35 °C. The results demonstrate that λ -cyhalothrin exhibits temperature-dependent efficacy within a 7-day exposure period, with higher temperatures resulting in increased mortality rates for both tested life stages. For larvae, mortalities ranged 81.1 (20 °C)–94.4% (35 °C) (half-label dose in the absence of food), 90.0 (20 °C)–100.0% (35 °C) (label dose in the absence of food), 72.2 (20 °C)–85.6% (35 °C) (half-label in the presence of food), 88.9 (20 °C)–100.0% (35 °C) (label dose in the presence of food) 7 days post-exposure. For adults, mortalities ranged 35.6 (20 °C)–74.4% (35 °C) (half-label dose in the absence of food), 43.3 (20 °C)–97.8% (35 °C) (label dose in the absence of food), 24.4 (20 °C)–65.6% (35 °C) (half-label in the presence of food), 38.9 (20 °C)–94.4% (35 °C) (label dose in the presence of food) after 7 days of exposure. Additionally, the significance of food substrate presence in absorbing and providing a barrier against insecticidal toxicity is highlighted, as it negatively impacted mortality rates. Overall, these findings elucidate the complex interactions among temperature, dosage, and environmental factors that optimize λ -cyhalothrin administration efficiently managing *A. diaperinus*.

1. Introduction

The lesser mealworm, or darkling beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), is a significant secondary pest of stored products worldwide (Sammarco et al., 2023). The importance of managing this pest is increasing due to its ubiquitous presence in the poultry industry, its implications for human and animal health, and the emerging challenges for its effective control (Smith et al., 2022). The completion of life cycle of *A. diaperinus* depends on temperature, ranging from 164.24 days at 20 °C to 28.74 days at 35 °C (Rueda and Axtell, 1996), thus forming poultry houses an ideal environment for its development.

The documented role of lesser mealworms as vectors for various pathogens and parasites is extensive. Numerous bacteria, including *Micrococcus* spp., *Streptococcus* spp., *Campylobacter jejuni* (Jones and Little) Veron & Chatelain (Campylobacteriales: Campylobacteriaceae),

Staphylococcus spp., *Pseudomonas* spp., and *Salmonella* spp., have been isolated from *A. diaperinus* (Hazeleger et al., 2008; Agabou and Alloui, 2010; Cucini et al., 2020). Additionally, this insect is known to be an efficient reservoir for tapeworms, turkey enterovirus, avian leukosis virus (ALVs), and infectious bursal disease virus (IBDV) (Agabou and Alloui, 2010; Leffer et al., 2010; Dinev, 2013; Smith et al., 2022). Several research studies have highlighted the economic consequences of chickens consuming larvae and adults of *A. diaperinus*, resulting in decreased animal weight and growth (Hickmann et al., 2018; Japp et al., 2010; Smith et al., 2022). Various bacterial pathogens that are spread in poultry facilities can be further transmitted to humans (Tamburro et al., 2021). Recently, a rare instance of gastrointestinal canthariasis linked to the ingestion of *A. diaperinus* larvae has been reported. Patients affected by this condition experienced symptoms such as vomiting and intermittent diarrhea, with little to no relief even after receiving medication (Tilak et al., 2023).

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Over the last two decades, pyrethroids and organophosphates have been extensively employed to protect stored products against various pests (Nayak et al., 2002; Ghimire et al., 2016; Yao et al., 2019). In the poultry industry, chemical strategies are frequently utilized to manage *A. diaperinus* populations. Nevertheless, conventional insecticides exhibit limited efficacy in achieving satisfactory control, particularly when beetle populations reach epidemic proportions. Moreover, *A. diaperinus* has demonstrated resistance to certain pyrethroids. In a study by Renault and Colinet (2021), which assessed permethrin and cyfluthrin resistance in 10 populations of *A. diaperinus* from French poultry farms, LT_{50} values were found to be 10 times higher than in susceptible insects for at least half of the farm populations. Hickmann et al. (2018) observed significant variation in susceptibility to cypermethrin and chlorpyrifos among adult populations of *A. diaperinus* in southern Brazil. The LC_{50} values for cypermethrin and chlorpyrifos ranged from 0.46 to 4.93 μg active ingredient (a.i.)/ cm^2 and 0.17–5.30 μg a.i./ cm^2 , respectively, indicating 10- and 31-fold resistance.

Despite the growing issue of resistance and the limited availability of registered chemical products for managing *A. diaperinus*, recent research efforts focused on evaluating various contact insecticides, either independently or in combination, also investigating factors that influence their efficacy (Kavallieratos et al., 2022a, 2022b, 2023). Kavallieratos et al. (2022a) provided insights into the lethal effects of deltamethrin, etofenprox, and the combination of piperonyl butoxide + acetamiprid + d-tetramethrin against *A. diaperinus* larvae. The presence of food affected the mortality levels, concerning the last two insecticides in contact bioassays. Furthermore, the type of surface plays a major role in the efficacy of the pyrethroid etofenprox, with treatments on glass demonstrating superior effectiveness against larvae and adults of *A. diaperinus* compared to ceramic, plastic, and wood-treated surfaces (Kavallieratos et al., 2023).

The synthetic pyrethroid, λ -cyhalothrin, is renowned for its widespread use as a highly effective insecticide for mosquito control (He et al., 2008; Pettit et al., 2010; Raghavendra et al., 2011; Aydogdu et al., 2017). Besides its notable contribution to public health, λ -cyhalothrin serves as a pivotal tool in agricultural practices, offering effective management solutions for a diverse range of pests encompassing Lepidoptera, Hemiptera, and Coleoptera (Elhalwagy and Zaki, 2009; Whalen et al., 2012; Keyhanian et al., 2021), while it exhibits significantly lower toxicity to mammals compared to organophosphorus compounds (Zhu et al., 2020). Derived from the natural insecticide pyrethrin found in chrysanthemum flower heads, λ -cyhalothrin engages with sodium (Na^+) channels in nerve membranes. This interaction is characterized by an extension of the channels' opening duration, crucial for responding to nerve stimuli. Upon reaching the active site, the toxin induces uncontrolled nerve discharges, manifesting as twitches or tremors and, in severe cases, leading to paralysis. This mode of action highlights the potency of λ -cyhalothrin in disrupting the neurological functions of the target insects (Martins-Gomes et al., 2022).

While λ -cyhalothrin has demonstrated promising insecticidal activity, the available data on its utilization against *A. diaperinus* is currently limited (Yeasmin et al., 2015; Rice and Lambkin, 2009). Moreover, a notable gap exists in the literature concerning the performance of pyrethroids, particularly λ -cyhalothrin, under different temperature conditions when applied against *A. diaperinus*. Thus, the current study underscores the insecticidal potential of λ -cyhalothrin, especially in the context of a temperature-dependent frame against *A. diaperinus*, providing essential insights and valuable information for enhancing pest control strategies.

2. Materials and methods

2.1. Insects and food

Alphitobius diaperinus was reared in darkness at 30 °C and 55% relative humidity (RH) (Rice and Lambkin, 2009) in the Laboratory of

Agricultural Zoology and Entomology at the Agricultural University of Athens, Greece. The insects were provided with a food source consisting of a mixture of yeast (25%) and wheat bran (75%), supplemented with additional apple pieces to maintain optimal moisture content (Rice and Lambkin 2009). Unsexed, medium-sized larvae (7 mm) and adult beetles (<7 days old), randomly collected from the insect culture, were used in the experiments.

2.2. Formulations

The commercial formulation ICON 10 CS (Syngenta, Greece) was used for the experiments, containing λ -cyhalothrin active ingredient (a. i.) at 10.7 w/w. Two doses of the formulation were tested: the half-label dose (0.0226 mg a.i./ cm^2) and the label dose (0.0452 mg a.i./ cm^2) for surface treatments.

2.3. Bioassays

For the experiment, three groups of Petri dishes ($\varnothing$ 8 cm $\times$ 1.5 cm; surface area 50.27 cm^2), each consisting of three sub-replicates, were prepared for both adults and larvae. Each sub-replicate consisted of 10 dishes intended to contain one individual per dish to mitigate cannibalism (Szczepanik et al., 2016). At least 24 h before the initiation of the experiment, the bottom of each dish was lined with cement (CEM I 52.5 N material, Durostick, Greece), while its inner vertical surface was treated with polytetrafluoroethylene (Sigma-Aldrich Chemie GmbH, Germany) to prevent insects from escaping. Each dish received 1 mL of the insecticide solution at either the half-label or label dose. The application was conducted using an airbrush (BD-134K, Fengda, UK) to ensure a fine mist. Subsequently, the treated dishes were allowed to dry for 2 h at ambient air temperature. To examine whether the presence of food affects the mortality of *A. diaperinus* adults and larvae, two sets of experiments were performed. In the first set, each dish received 0.5 g of insect diet, weighed using a Precisa XB3200D balance (Alpha Analytical Instruments, Greece). In the second set, no insect diet was added. As controls, an equivalent number of dishes with and without food, receiving no treatment, were prepared. To investigate the impact of temperature variation on the response of *A. diaperinus* adults and larvae to insecticide exposure, bioassays were conducted at temperatures of 20 °C, 25 °C, 30 °C, and 35 °C, RH 55%, and 0 L: 24 D light-dark photoperiod inside incubators. Mortality was assessed at one, three, five, and seven days post-exposure. Insects were considered dead if they showed no movement upon being touched with the bristles of a fine-hair, round-tip brush, observed under an Olympus SZX9 stereomicroscope (Bacacos S.A., Greece). A total of 2160 adults or larvae of *A. diaperinus* were utilized in the experiment, comprising 3 replicates $\times$ 3 sub-replicates $\times$ 10 individuals $\times$ 3 doses (half-label dose, label dose, control) $\times$ 4 temperatures $\times$ 2 scenarios (with and without food).

2.4. Data analysis

Since the mortality rate in the control trials was below 5% (Tables S1–S4), data correction was deemed unnecessary. Subsequently, the data underwent a $\log(x + 1)$ transformation to standardize variance (Zar, 2014; Scheff and Arthur, 2018). Mortality data for each developmental stage were subjected to separate repeated-measures multivariate analysis of variance (MANOVA) (Sall et al., 2001). Exposure served as the repeated factor, with mortality as the response variable. Dose, presence or absence of food, and temperature were the main effects, with interactions among these factors also included in the analysis. Post-hoc analysis of means at a significance level of 5% was conducted using the Tukey test (HSD) (Sokal and Rohlf, 1995). All statistical analyses were carried out using JMP 16.2 software (SAS Institute Inc, 2021).

3. Results

Between exposures, all main effects and the dose $\times$ temperature interaction were significant for larvae and adults of *A. diaperinus*. Within exposures, the exposure $\times$ dose, exposure $\times$ temperature, and exposure $\times$ dose $\times$ temperature interactions, and exposure were significant for both *A. diaperinus* larvae and adults (Table 1). *Alphitobius diaperinus* larvae demonstrated significantly higher mortality as temperature increased both at half-label and label doses of λ -cyhalothrin in the presence and absence of food, mainly between 20–25 °C and 30–35 °C. Additionally, larval mortality increased significantly with higher doses at low temperatures, while at high temperatures, the increase in mortality with rising doses was generally not significant. Mortality also increased with time irrespective of temperature and dose applied. Food deprivation resulted in higher mortalities in *A. diaperinus* larvae, with respect to the temperature and dose applied (Tables 2 and 3).

Specifically, in the absence of food, the half-label dose of λ -cyhalothrin caused low mortality in *A. diaperinus* larvae after one day of exposure at 20 °C (2.2%) to correspondingly moderate mortality rates at 35 °C (23.3%). Nevertheless, significantly higher mortality rates were exhibited after three days of exposure at the same dose for all temperatures (34.4–80%, respectively), ultimately reaching 94.4% mortality on the final day of exposure at 35 °C. The recommended dose of λ -cyhalothrin caused low to moderate mortality after one day of exposure at all temperatures tested (11.1–27.8%). Mortality increased significantly at three days of exposure, exhibiting high rates ranging from 52.2% at 20 °C to 88.9% at 35 °C. Complete mortality was observed for larvae treated with the label dose after five days of exposure at 30 and 35 °C, as well as seven days post-exposure at 25 °C (Table 2). A similar trend was observed for the larvae exposed to λ -cyhalothrin with the presence of the food source, although mortalities were slightly lower. Half-label dose of the insecticide resulted in low mortalities of *A. diaperinus* larvae after one day of exposure, while mortalities significantly increased after three days of exposure (28.9–78.9% for 20–35 °C), reaching 85.6% mortality at seven days post-exposure at 35 °C. The label dose of λ -cyhalothrin caused low to moderate mortalities at the initial exposure, which significantly rose after three days of exposure (6.7–23.3% and 47.8–85.6%, respectively). Complete mortality was observed for larvae treated with the label dose at 30 and 35 °C after five days of exposure (Table 3).

Table 1

MANOVA parameters for the main effects and associated interactions for the mortality levels of *Alphitobius diaperinus* adults and larvae between and within exposure intervals (error df = 128 for both developmental stages).

Effect	Adults			Larvae	
	df	F	P	F	P
Between exposure intervals					
Source					
Intercept	1	4362.5	<0.01	19,260.9	<0.01
Dose	1	45.7	<0.01	68.1	<0.01
Temperature	3	97.1	<0.01	43.3	<0.01
Food	1	14.0	<0.01	6.6	0.01
Dose $\times$ temperature	3	5.3	0.01	3.6	0.02
Dose $\times$ food	1	0.1	0.76	0.7	0.41
Temperature $\times$ food	3	0.3	0.80	0.1	0.96
Dose $\times$ temperature $\times$ food	3	0.2	0.92	0.1	0.94
Within exposure intervals					
Source	df	F	P	F	P
Exposure	3	3956.1	<0.01	287.8	<0.01
Exposure $\times$ dose	3	8.3	<0.01	15.2	<0.01
Exposure $\times$ temperature	9	22.7	<0.01	15.3	<0.01
Exposure $\times$ dose $\times$ temperature	9	4.4	<0.01	3.8	0.01
Exposure $\times$ food	3	2.4	0.07	1.1	0.36
Exposure $\times$ dose $\times$ food	3	1.2	0.30	0.1	0.98
Exposure $\times$ temperature $\times$ food	9	0.8	0.62	0.4	0.92
Exposure $\times$ dose $\times$ temperature $\times$ food	9	0.4	0.94	0.5	0.86

Concerning *A. diaperinus* adults, mortality increased with time irrespective of temperature, presence/absence of food, and dose, however, on the first day of exposure no mortality was observed, except for low mortalities at 35 °C when the label dose was applied. Adults demonstrated higher mortality as temperature increased both at half-label and label dose of λ -cyhalothrin in the presence and absence of food. The increment in the dose applied led to higher mortalities both in the presence and absence of food, but significant differences were only observed after seven days of exposure. Higher mortality rates in *A. diaperinus* adults were noted under the food deprivation scenario, influenced by temperature and dose (Tables 4 and 5). Specifically, in the absence of food, *A. diaperinus* adults were not affected by the insecticide at a half-label dose after one day of exposure. At three days post-exposure, mortality was low but significantly increased after five days of exposure, reaching 53.3% at 35 °C. The maximum mortality (74.4%) was observed at seven days post-exposure at 35 °C. The label dose of λ -cyhalothrin resulted in zero to low mortalities (3.3% at 35 °C). A significant increase in mortality was observed from three (1.1–32.2%) to five days post-exposure (12.2–80.0%), ultimately reaching 43.3–97.8% after seven days of exposure for 20–35 °C (Table 4). Lower mortalities were observed for *A. diaperinus* adults exposed to λ -cyhalothrin with the presence of food, following a similar pattern. At half-label dose, adults exposed for one and three days to the insecticide were not or little affected by the treatment (0.0% and 0.0–4.4%, respectively). Mortality significantly increased at five days of exposure, with respect to the temperature rise (3.3–52.2%), reaching 24.4–65.6% after seven days of exposure for 20–35 °C, respectively. Concerning the label dose of λ -cyhalothrin, higher mortalities were observed after three days of exposure, increasing with the increment of temperature (0.0–26.7% for 20–35 °C, respectively). Mortalities further increased with exposure and temperature rise, reaching 8.9–74.4% at five and 38.9–94.4% at seven days of exposure for 20–35 °C, respectively (Table 5).

4. Discussion

Temperature exerts a multifaceted influence on pesticide toxicity, with its impact varying depending on several factors such as the mode of action of the insecticide, the target insect species, or the route of exposure (ingestion or contact) (Laskowski et al., 2010; Glunt, 2013). Temperature changes typically alter insecticide processes such as absorption, persistence, and degradation, as well as the sensitivity of insect pest species (Li et al., 2024). Although pyrethroids and other insecticides display a variable response to post-exposure temperature, there is generally a negative association with temperature, meaning their toxicity decreases with higher temperatures (Arthur, 1996). Boukouvala and Kavallieratos (2022) found that increasing temperature negatively affected the efficacy of etofenprox in both larvae and adults of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae). Similarly, cyfluthrin demonstrated a negative temperature coefficient for *T. confusum*, *Prostephanus truncatus* (Horn) (Coleoptera: Bostrychidae), *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), and *Cadra cautella* (Walker) (Lepidoptera: Pyralidae) (Subramanyam and Cutkomp, 1987). In the same study, bioallethrin was also associated with a negative temperature coefficient for *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) and *P. interpunctella* (Subramanyam and Cutkomp, 1987). As temperature rises, it causes an elevation of the metabolic rate and alters behavioral and physiological characteristics, as well as increasing detoxification processes due to stress (Clarke and Fraser, 2004; Gonza'lez-Tokman et al., 2020). High-temperature conditions usually induce strong tolerance mechanisms in certain insect species. For example, exposure of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) to 31 °C induced the activity of detoxifying enzymes like cytochrome P450 (CYP450), increasing the tolerance to thiamethoxam (Guo et al., 2018). Similarly, higher temperatures reduced the recorded mortality of *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae), which is

Table 2

Mean (%) mortality $\pm$ standard errors (SE) of *Alphitobius diaperinus* larvae treated with λ -cyhalothrin after 1, 3, 5, and 7 days in the absence of food. For each column, means followed by the same lowercase letters are not significantly different (df = 7, 71; Tukey HSD test at $P = 0.05$). For each row, means followed by the same uppercase letters are not significantly different (df = 3, 35; Tukey HSD test at $P = 0.05$).

Dose	Temperature	Exposure (days)				F	P
		1	3	5	7		
Half-label	20 °C	2.2 $\pm$ 1.5 Cc	34.4 $\pm$ 4.1 Bd	67.8 $\pm$ 5.5 ABc	81.1 $\pm$ 3.9 Ac	88.0	<0.01
	25 °C	6.7 $\pm$ 2.4 Bbc	37.8 $\pm$ 3.2 Acd	73.3 $\pm$ 2.9 Abc	85.6 $\pm$ 4.1 Abc	36.9	<0.01
	30 °C	15.6 $\pm$ 4.7 Babc	63.3 $\pm$ 2.9 Aab	83.3 $\pm$ 2.4 Aab	87.8 $\pm$ 2.8 Aabc	18.6	<0.01
	35 °C	23.3 $\pm$ 4.1 Ba	80.0 $\pm$ 2.9 Aa	94.4 $\pm$ 2.4 Aa	94.4 $\pm$ 2.4 Aab	56.6	<0.01
Label	20 °C	11.1 $\pm$ 4.2 Babc	52.2 $\pm$ 7.0 Abc	73.3 $\pm$ 5.8 Abc	90.0 $\pm$ 2.9 Aabc	23.7	<0.01
	25 °C	20.0 $\pm$ 3.3 Bab	77.8 $\pm$ 4.9 Aa	97.8 $\pm$ 1.5 Aa	100.0 $\pm$ 0.0 Aa	23.1	<0.01
	30 °C	22.2 $\pm$ 2.2 Ba	86.7 $\pm$ 5.0 Aa	100.0 $\pm$ 0.0 Aa	100.0 $\pm$ 0.0 Aa	139.6	<0.01
	35 °C	27.8 $\pm$ 4.6 Ba	88.9 $\pm$ 3.1 Aa	100.0 $\pm$ 0.0 Aa	100.0 $\pm$ 0.0 Aa	50.5	<0.01
	F	7.1	20.9	13.4	7.1		
	P	<0.01	<0.01	<0.01	<0.01		

Table 3

Mean (%) mortality $\pm$ standard errors (SE) of *Alphitobius diaperinus* larvae treated with λ -cyhalothrin after 1, 3, 5, and 7 days with the presence of food. For each column, means followed by the same lowercase letters are not significantly different (df = 7, 71; Tukey HSD test at $P = 0.05$). For each row, means followed by the same uppercase letters are not significantly different (df = 3, 35; Tukey HSD test at $P = 0.05$).

Dose	Temperature	Exposure (days)				F	P
		1	3	5	7		
Half-label	20 °C	1.1 $\pm$ 1.1 Cc	28.9 $\pm$ 4.2 Bd	61.1 $\pm$ 4.2 Ac	72.2 $\pm$ 3.6 Ac	134.6	<0.01
	25 °C	4.4 $\pm$ 1.8 Cbc	31.1 $\pm$ 3.9 Bd	64.4 $\pm$ 5.3 ABc	77.8 $\pm$ 5.2 Abc	42.6	<0.01
	30 °C	8.9 $\pm$ 2.6 Babc	53.3 $\pm$ 3.3 Abc	77.8 $\pm$ 3.6 Abc	84.4 $\pm$ 5.0 Aabc	31.2	<0.01
	35 °C	20.0 $\pm$ 5.5 Bab	78.9 $\pm$ 4.6 Aab	85.6 $\pm$ 2.9 Aab	85.6 $\pm$ 2.9 Aabc	15.7	<0.01
Label	20 °C	6.7 $\pm$ 3.3 Bbc	47.8 $\pm$ 4.5 Ac	68.9 $\pm$ 5.4 Ac	88.9 $\pm$ 3.1 Aab	37.7	<0.01
	25 °C	16.7 $\pm$ 1.7 Ca	58.9 $\pm$ 3.9 Babc	87.8 $\pm$ 2.8 Aab	92.2 $\pm$ 3.2 Aab	136.2	<0.01
	30 °C	18.9 $\pm$ 2.0 Ba	82.2 $\pm$ 4.3 Aa	100.0 $\pm$ 0.0 Aa	100.0 $\pm$ 0.0 Aa	170.0	<0.01
	35 °C	23.3 $\pm$ 2.9 Ba	85.6 $\pm$ 2.9 Aa	100.0 $\pm$ 0.0 Aa	100.0 $\pm$ 0.0 Aa	125.5	<0.01
	F	9.2	21.7	13.3	7.4		
	P	<0.01	<0.01	<0.01	<0.01		

Table 4

Mean (%) mortality $\pm$ standard errors (SE) of *Alphitobius diaperinus* adults treated with λ -cyhalothrin after 1, 3, 5, and 7 days in the absence of food. For each column, means followed by the same lowercase letters are not significantly different (df = 7, 71; Tukey HSD test at $P = 0.05$). For each row, means followed by the same uppercase letters are not significantly different (df = 3, 35; Tukey HSD test at $P = 0.05$). Where no letters exist, no significant differences were noted.

Dose	Temperature	Exposure (days)				F	P
		1	3	5	7		
Half-label	20 °C	0.0 $\pm$ 0.0 B	0.0 $\pm$ 0.0 Bc	4.4 $\pm$ 2.9 Bb	35.6 $\pm$ 5.3 Ac	47.9	<0.01
	25 °C	0.0 $\pm$ 0.0 B	1.1 $\pm$ 1.1 Bbc	37.8 $\pm$ 4.0 Aa	53.3 $\pm$ 3.3 Abc	211.1	<0.01
	30 °C	0.0 $\pm$ 0.0 B	4.4 $\pm$ 2.4 Bbc	38.9 $\pm$ 3.9 Aa	54.4 $\pm$ 4.7 Abc	74.2	<0.01
	35 °C	0.0 $\pm$ 0.0 C	6.7 $\pm$ 2.4 Bbc	53.3 $\pm$ 7.8 Aa	74.4 $\pm$ 2.9 Aab	73.0	<0.01
Label	20 °C	0.0 $\pm$ 0.0 C	1.1 $\pm$ 1.1 Cbc	12.2 $\pm$ 3.2 Bb	43.3 $\pm$ 4.7 Ac	45.8	<0.01
	25 °C	0.0 $\pm$ 0.0 B	3.3 $\pm$ 1.7 Bbc	40.0 $\pm$ 4.4 Aa	70.0 $\pm$ 3.3 Aab	99.9	<0.01
	30 °C	0.0 $\pm$ 0.0 C	11.1 $\pm$ 4.2 Bb	54.4 $\pm$ 4.4 Aa	87.8 $\pm$ 4.3 Aa	59.7	<0.01
	35 °C	3.3 $\pm$ 2.4 C	32.2 $\pm$ 6.0 Ba	80.0 $\pm$ 6.7 Aa	97.8 $\pm$ 1.5 Aa	64.6	<0.01
	F	2.3	9.3	19.6	13.7		
	P	0.04	<0.01	<0.01	<0.01		

linked to the elevated expression activity of the CYP450 enzyme family (Wang et al., 2021).

Conversely, several insecticides, including certain pyrethroids, demonstrate improved efficacy with increasing temperatures. For example, a combination of acetamiprid, d-tetramethrin, and piperonyl butoxide increased mortality rates for *Trogoderma granarium* Everts (Coleoptera: Dermestidae) at 35 °C compared to 30 °C (Kavallieratos and Boukouvala, 2019). Additionally, a recent study on the effect of chlorantraniliprole on *P. truncatus* revealed a similar mortality pattern (Boukouvala and Kavallieratos, 2021). This positive correlation between temperature and insecticide efficacy has also been observed in compounds used against various other pest species, including *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *Anopheles funestus* Giles (Diptera: Culicidae), where the toxicity of compounds such as λ -cyhalothrin, methomyl, and bendiocarb increased with rising

temperatures (Glunt et al., 2018; Ismail, 2023). δ -Phenothrin exhibited a positive temperature coefficient for *T. confusum* (Subramanyam and Cutkomp, 1987). Karimzadeh et al. (2020) reported that the toxic effect of cypermethrin was enhanced at higher temperatures against *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae). Pra'zi'c Goli'c et al. (2016) also demonstrated that the efficacy of deltamethrin increased with rising temperatures when applied against *S. oryzae*. Additionally, Uddin and Ara (2006) observed a marked decrease in the effectiveness of cypermethrin and λ -cyhalothrin, along with other insecticides, against *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) as temperatures lowered.

However, in some instances, temperature does not significantly impact insecticide activity. For example, Amarasekare and Edelson (2004) found that esfenvalerate provided complete mortality against grasshoppers across different temperature values, varying from 10 to 35

Table 5

Mean (%) mortality $\pm$ standard errors (SE) of *Alphitobius diaperinus* adults treated with λ -cyhalothrin after 1, 3, 5, and 7 days with the presence of food. For each column, means followed by the same lowercase letters are not significantly different (df = 7, 71; Tukey HSD test at $P = 0.05$). For each row, means followed by the same uppercase letters are not significantly different (df = 3, 35; Tukey HSD test at $P = 0.05$). Where no letters exist, no significant differences were noted.

Dose	Temperature	Exposure (days)				F	P
		1	3	5	7		
Half-label	20 °C	0.0 $\pm$ 0.0 B	0.0 $\pm$ 0.0 Bb	3.3 $\pm$ 1.7 Bc	24.4 $\pm$ 2.9 Ae	49.1	<0.01
	25 °C	0.0 $\pm$ 0.0 B	0.0 $\pm$ 0.0 Bb	28.9 $\pm$ 3.9 Ab	38.9 $\pm$ 3.9 Ad	497.3	<0.01
	30 °C	0.0 $\pm$ 0.0 B	1.1 $\pm$ 1.1 Bb	33.3 $\pm$ 3.7 Aab	45.6 $\pm$ 2.9 Acd	188.8	<0.01
	35 °C	0.0 $\pm$ 0.0 C	4.4 $\pm$ 2.4 Bb	52.2 $\pm$ 4.0 Aab	65.6 $\pm$ 4.1 Abc	89.3	<0.01
Label	20 °C	0.0 $\pm$ 0.0 C	0.0 $\pm$ 0.0 Cb	8.9 $\pm$ 2.6 Bc	38.9 $\pm$ 2.0 Ad	61.4	<0.01
	25 °C	0.0 $\pm$ 0.0 C	2.2 $\pm$ 1.5 Cb	22.2 $\pm$ 4.3 Bb	56.7 $\pm$ 4.1 Abc	92.7	<0.01
	30 °C	0.0 $\pm$ 0.0 B	5.6 $\pm$ 4.4 Bb	36.7 $\pm$ 3.7 Aab	68.9 $\pm$ 3.5 Aab	77.8	<0.01
	35 °C	1.1 $\pm$ 1.1 C	26.7 $\pm$ 3.3 Ba	74.4 $\pm$ 6.7 Aa	94.4 $\pm$ 1.8 Aa	153.4	<0.01
	F	1.0	15.1	24.5	28.8		
	P	0.44	<0.01	<0.01	<0.01		

°C under laboratory settings. Additionally, in the same study, the efficacy of azadirachtin proved unaffected by changing temperature conditions. Another study examined the activity of several insecticides against non-target organisms, such as *Bracon nigricans* (Szépligeti) (Hymenoptera: Braconidae) (Abbes et al., 2015). It was discovered that toxicity of spinosad was not considerably affected by temperature changes. These results imply that whether or not temperature influences efficacy depends critically on the mode of action and the exposure route of the exposed insect species (Abbes et al., 2015). Although related research efforts support both negative and positive temperature coefficients for pyrethroids (Subramanyam and Cutkomp, 1987; Karimzadeh et al., 2020; Pražák et al., 2016), our study demonstrated that λ -cyhalothrin provided increased mortality rates for both life stages of *A. diaperinus* with higher temperatures. These findings underscore the importance of considering temperature as a critical factor in optimizing the application of pyrethroids for controlling stored-product insects.

The current study demonstrates distinct responses of both larvae and adult stages to λ -cyhalothrin exposure. The differential mortality values observed between the larval and adult stages of *A. diaperinus* when exposed to these treatments highlight the varying susceptibilities across insect life stages. Specifically, exposed larvae exhibited higher mortality rates than adults under the same treatment conditions. Contrary to our findings, various insecticidal formulations, including pyrethroids, demonstrated higher susceptibility in treated adults than larvae. For instance, Athanassiou et al. (2015) reported that the larvae of *T. granarium* and *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) exhibited higher tolerance, while adults of both species were more susceptible when exposed to concrete treated with α -cypermethrin. Additionally, cypermethrin, deltamethrin, pirimiphos-methyl, silicoSec, s-methoprene, and spinosad were able to suppress *T. granarium* adults even at the lowest dose; however, these insecticides were ineffective against *T. granarium* larvae (Kavallieratos et al., 2017). Furthermore, pirimiphos-methyl, deltamethrin, spinosad, and silicoSec were found more effective against adults of *T. molitor* than against larvae (Kavallieratos et al., 2019). In contrast, the monoterpenes α -pinene, α -phellandrene, eugenol, α -humulene, and caryophyllene oxide exhibited higher mortality to larvae than to adults of *T. molitor* in toxicity tests two days after exposure (Martínez et al., 2018). The authors hypothesized that differences in the penetration and metabolism of these compounds within the bodies of adults and larvae could account for the varying levels of observed toxicity (Martínez et al., 2018). The disparity among insect developmental stages can be attributed to several physiological and behavioral factors inherent to each life stage (Lockwood et al., 1984). Specifically, the larval epidermis shows differences in thickness, hardness, and lipid composition. It is generally thinner and less developed compared to the hardened exoskeletons of adults, making larvae more vulnerable to penetration by any active ingredient (Mewis and Ulrichs, 2001; Andersen, 2010; Panini et al., 2016; Doucet and Retnakaran, 2016). Pyrethroids are lipophilic compounds that dissolve

into the outer wax layer of the cuticle and then partition into its inner polar layers. This penetration through the cuticle occurs via a network of wax canals, accessing intersegmental areas or the lipophilic tracheal lining through the spiracles (Noble-Nesbitt, 1970; Antwi and Reddy, 2015). Holometabolous insects typically exhibit higher lipid content during their larval stage than their adult stage (Dossey et al., 2016; dos Santos Aguilar, 2021), potentially explaining the higher larval susceptibility compared to adults demonstrated in the current research.

The neurotoxic activity of pyrethroids, including λ -cyhalothrin, is generally expected to increase adult mobility, resulting in increased exposure and a higher insecticidal efficacy (Alzogaray et al., 1997; Guedes et al., 2008). In the current study, adults were less susceptible to the pyrethroid tested, contradicting the above consensus. This exception has also been documented for psocid species, *Liposcelis bostrychophila* Badonnel and *L. entomophila* (Enderlein) (Psocoptera: Liposcelidae), for which reduced mobility was documented when treated with PyGanic Pro SC on concrete surfaces, attributed to a possible weak repellence of the pyrethrin to the tested insect species (Guedes et al., 2008).

From another perspective, the detoxification of insects plays an important role in the effectiveness of an insecticide at a particular life stage. Detoxification enzymes work by catalyzing chemical reactions that modify or break down toxic compounds into less harmful forms, allowing organisms to mitigate the toxic effects of pesticides and other xenobiotics (Heckel, 2014). Pedersen et al. (2020) revealed that adults of *T. molitor* exhibit significantly higher activity of detoxification enzymes such as cytochrome P450, esterases (EST), and glutathione S-transferase (GST) compared to larvae when treated with α -cypermethrin. If this holds for *A. diaperinus*, lower susceptibility of adults to λ -cyhalothrin would be expected compared to larvae, as exhibited here. Nevertheless, further comprehensive research is necessary to thoroughly investigate all contributing factors influencing this outcome before drawing definitive conclusions.

In both adult and larval stages of *A. diaperinus*, mortality rates were higher in the absence of food compared to conditions with food present. This observation underscores the influence of food presence on the efficacy of insecticides, which can potentially degrade or absorb them, thereby creating a protective barrier between the treated surface and the targeted insects (Arthur, 2000). Additional studies by Arthur (2015a, 2015b) have demonstrated that insecticides such as chlorfenapyr and beta-cyfluthrin exhibit reduced effectiveness against *T. castaneum* in the presence of food, primarily due to their absorption by food particles, resulting in increased survival rates. Moreover, concerning *A. diaperinus*, Kavallieratos et al. (2022a) have noted that the presence of bran flakes appears to reduce larval mortality induced by the tested insecticides. This is more evident when a diet exhibiting a higher absorbency, such as flour, is provided (Kharel et al., 2014).

Concerning the mortality rates of *A. diaperinus* larvae and adults, when different doses of λ -cyhalothrin were administered, both life stages exhibited higher mortality as the insecticidal dose increased. This has

been previously documented for several pyrethroid insecticides against *A. diaperinus*, including deltamethrin (Kavallieratos et al., 2022b), cypermethrin (Oliveira et al., 2016), cyfluthrin (Hamm et al., 2006), and bifenthrin (Boozer, 2008). Reducing application rates aims to address the problem of resistance development in storage pests, particularly against organophosphates and pyrethroids, which have been extensively used for pest management (Kumar and Kalita, 2017; Attia et al., 2020). The significance of the findings of the current study lies in the fact that λ -cyhalothrin, a pyrethroid, achieved high mortality rates in both larvae and adults of *A. diaperinus* even when a half-label dose was administered, highlighting its effectiveness and potential for resistance management.

Both larvae and adults of *A. diaperinus* exhibit varying susceptibilities to λ -cyhalothrin, influenced by factors beyond temperature alone such as exposure, life stage, administered insecticidal dose, and food presence, forming complex interplays. Exploring the underlying mechanisms driving these interactions will optimize the application methods of insecticidal formulations and enhance their efficacy. The current obtained knowledge is pivotal for developing integrated pest management (IPM) strategies that maximize efficacy and minimize environmental impact or operational costs. Further investigation into these puzzling issues will contribute to refining IPM approaches tailored to the specific challenges posed by *A. diaperinus*.

CRedit authorship contribution statement

Federica Lo Muzio: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nikoleta Eleftheriadou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Maria C. Boukouvala:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Constantin S. Filintas:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Data curation. **Demeter Lorenta S. Gidari:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Data curation. **Giacinto Salvatore Germinara:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Nickolas G. Kavallieratos:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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10. Insecticidal activity and persistence of a natural Cuban zeolite and a diatomaceous earth against *Sitophilus granarius* on wheat

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Insecticidal activity and persistence of a natural Cuban zeolite and a diatomaceous earth against *Sitophilus granarius* on wheat

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Abstract

Sitophilus granarius (L.) (Coleoptera, Curculionidae) is one of the main pest of stored wheat worldwide. Inert dusts can play an important role within Integrated Pest Management strategies (IPM). Diatomaceous earths are the most often tested materials for controlling stored-product pests; whilst, less information is available on zeolites. This study aimed to expand the knowledge on the insecticidal activity and persistence of a natural Cuban zeolite (NCZ) compared to a diatomaceous earth (DE). In insecticidal tests, grain samples were treated with 0.25, 0.5, 1, 2, 4, and 8 kg/ton of DE or NCZ and infested with 20 *S. granarius* adults. The number of dead specimens after 3, 7, 14, and 21 day-exposure and the total F1 progeny were recorded. In persistence tests, grain was treated with 0.25, 0.5, 1, 2, and 4 kg/ton of dusts, while infestation with *S. granarius* adults was started 3, 6, or 12 months after treatment. The NCZ treatment reached the total adult mortality at slightly higher doses and exposure times compared to DE. However, starting from 1 and 2 kg/ton doses for DE and NCZ respectively, the total insect mortality was reached within a short time and the mean progeny per replicate was below one individual. The persistence activity of NCZ and DE has been confirmed for up to 12 months from the treatment without significant differences between the two inert dusts. The NCZ appears of great interest for practical applications in controlling *S. granarius*.

Keywords: stored-product insect pests, inert dusts, mortality bioassays, physical control means, IPM.

Introduction

Wheat is one of the most important staple crops globally, characterized by consistently high consumer demand (Igrejas and Branlard 2020). In 2021, global wheat production exceeded 700 million tons (FAO, 2023), with Asia and Europe representing the regions contributing the largest shares to total world production (Le Gouis et al. 2020).

During storage, attacks of insect pests can result in substantial losses estimated at approximately 10-15% of the global annual production in developed countries (Rajendran 2002; Neethirajan et al. 2007) depending on climatic conditions, storage facilities and pest control measures (Rajendran 2020 and references therein; Tadesse 2020 a, b). The granary weevil, *Sitophilus granarius* (L.) (Coleoptera, Curculionidae) is a leading pest of wheat, barley, maize, rye, oats, and other grains during storage worldwide (Ahmad et al. 2021). The duration of the developmental stages of *S. granarius* differs according to season and environmental factors (Fouad et al. 2024). In general, female bores into the kernel and lay a single egg within the grain endosperm; then, four larval instars complete their development feeding into kernels and causing hidden infestation difficult to be timely detected and controlled (Rotundo et al. 2000).

Control of stored-product insect pests has been mainly based on the use of synthetic insecticides e.g., phosphine and methyl bromide (Hagstrum and Phillips 2017). However, due to their adverse impacts on human health and environment (Handford et al. 2015; Tudi et al. 2021), legislation limits on their use are increasing, especially in developed countries (Handford et al. 2015). Meanwhile, the rapid emergence of phosphine-resistant populations among major storage pest species has been reported globally (Nayak et al. 2020; Sakka et al. 2022; Machuca-Mesa et al. 2024). As a result, pest management in stored products has become increasingly difficult, driving global efforts toward the development of sustainable control tools for Integrated Pest Management (IPM) (Germinara et al. 2015; Rotundo et al. 2019; Chaudhari et al. 2021; Urrutia et al. 2021; Diab et al. 2025). In the last two decades, research on the use of inert dusts as sustainable control tools has gained increasing attention (Eroglu 2014; De Smedt et al. 2015). These materials are characterized by low mammalian toxicity and do not release chemical residues into food products (Ziaee et al. 2021 and references therein). On the other hand, insecticidal activity of different inert dusts has been proved against common storage pest species, such as *Sitophilus zeamais* Motschulsky, *S. oryzae* L., *Rhyzopertha dominica* (F.), *Tribolium* spp., *Callosobruchus maculatus* (Fabricius) (Rumbos et al. 2016; Lü et al. 2017; Eroglu et al. 2019; Gad et al. 2022; Baliota and

Athanassiou 2023; Milosavljević et al. 2024). However, to the best of our knowledge, the studies on inert dusts to control storage insect pests were mainly focused on diatomaceous earths (DEs), kaolin, sand, and various nanomaterials (Stadler et al. 2012; Islam et al. 2010; Campolo et al. 2014 a, b; Bougherra-Nehaoua et al. 2015; Bohinc et al. 2020; Zhanda et al. 2020). Whilst, less information is available on the insecticidal effect of other dusts, such as zeolites and attapulgite (Germinara et al. 2019; Karimzadeh et al. 2021; Lampiri et al. 2022; D'Isita et al. 2025).

Thus, in the present study, insectidal bioassays were carried out to evaluate the adult mortality and progeny production of *S. granarius* exposed to wheat kernels treated with increasing doses of NCZ or diatomaceous earth (DE) commercial formulation. Furthermore, persistence tests were performed for assessing long-term efficacy of both inert dusts.

Materials and Methods

Insects

Sitophilus granarius rearing was started using infested wheat kernels collected in storage facilities in Apulia region (Southern Italy). Insect colonies were reared on durum wheat (cv. Simeto) in cylindrical glass containers (Ø 15 × 15 cm) covered with a fine mesh net (0.5 mm) to allow air exchange. Rearing was maintained at 25±2°C, 60±5% relative humidity (R.H.), and continuous darkness, in incubators at the Laboratory of general and applied Entomology, Department of Agricultural Sciences, Food, Natural Resources and Engineering, University of Foggia. Insects of the eighth generation were used for laboratory bioassays

Inert dusts

Commercially available formulations of DE and NCZ powders were used in the experiments. The DE Insectosec (Newpharm, Padova, Italy) is a product composed of naturally occurring fossilized diatom frustules, contains 100% silicon dioxide, and a median particle size of approximately 8.2 µm. The NCZ Zeokill (Clinoptilolite-Heulandite 67.5%, Mordenite 32.5%) (Bioagrotech, Dogana, San Marino Republic) originated from the mechanical processing of a sedimentary rock of volcanic origin formed in Cuba and contains 65.65%

SiO₂, 11.02% Al₂O₃, 1.07% Na₂O, 1-74 K₂O, 2.68 CaO, 0.93 MgO, 0.09 BaO, 1.60 Fe₂O₃, and 0.26 TiO₂. The 93% of particle size is < 20 µm.

Insecticidal tests

Proper laboratory bioassays were set up to assess the insecticidal activity of the DE and NCZ against adults of *S. granarius*. Cylindrical plastic containers (Ø 6 x 8 cm), covered with screw caps with a central hole (2 cm) screened by a metallic net (mesh size 1 mm), were filled with 100 g of durum wheat (*cv.* Saragolla). Samples were uniformly treated with increasing doses (0.25, 0.5, 1, 2, 4, and 8 kg/ton) of DE or NCZ. Additional sets of untreated grains were used as controls. After treatment, each container was infested with 20 *S. granarius* adults (30-day-old) of mixed sexes and maintained at 25±2°C, 60±5% R.H., and L12:D12 photoperiod. Four replicates for each inert dust and dose were performed. After 3, 7, 14, and 21 days of exposure, the total number of dead specimens in each replicate was recorded and a mean adult mortality rate was calculated. Furthermore, after the 21-day mortality count, all insects were removed and kernel samples were maintained at the above-mentioned conditions. The total number of F1 progeny was recorded until no adults emerged for 5 consecutive days (Shazali, 1987).

Persistence tests

Samples (100 g) of durum wheat (*cv.* Saragolla) were placed in plastic containers similar to those described above and treated with 0.25, 0.5, 1, 2, and 4 kg/ton of DE or NCZ. Three different sets consisting of 3 replicates, for each dose and powder, were performed. Each replicate was infested with 20 *S. granarius* adults (30-day-old) 3, 6, or 12 months after treatment to test the persistence of the insecticidal activity of both inert dusts. Samples were maintained at 25±2°C, 60±5% R.H., and L12:D12 photoperiod. After 3, 7, 14, and 21 days of exposure, the total number of dead specimens in each replicate was recorded and used to calculate the mean adult mortality rate. For each set, the total number of F1 progeny was recorded similarly to that described above.

Statistical analysis

Data of mortality from insecticidal tests were first analyzed using a Generalized Linear Mixed Model (GLMM) to test the efficacy of the treatments on adult mortality. As predictor variables, the number of dead individuals on total samples was used resulting in “1” when

dead, and “0” for alive. Thus, a Binomial distribution was used checking for the effect of inert dust and dose with replicate membership as a random effect. Post-hoc analyses with Bonferroni correction were performed using the estimated marginal means to test the effect of each variable.

All data were submitted to Shapiro-Wilk’s test to verify normal distribution and to Levene’s test for homogeneity of variance. Since these assumptions were not met, the Kruskal-Wallis Test at $P < 0.05$ followed by nonparametric comparisons for all pairs using Dunn’s multiple comparison test with Bonferroni correction were performed to compare the number of dead insects between the two inert dusts and the control within each dose and time of exposure. This procedure was adopted for data from insecticidal tests, persistence tests, and to compare the insecticidal activity of 1 kg/ton DE and 2 kg/ton NCZ when infestation occurred at different times after treatment. To compare the mean number of F1 progeny emerged from untreated (control) or treated wheat with different doses of each inert dust in insecticidal tests, the Kruskal-Wallis Test ($P < 0.05$) followed by Dunn’s multiple comparison test with Bonferroni correction were performed. The F1 progeny emerged from wheat treated with the two inert dusts within each dose both in insecticidal and persistence tests were compared using the pairwise Mann-Whitney U test ($P < 0.05$) followed by Dunn’s comparison test with Bonferroni correction. The GLMM statistical analysis was conducted in JMP 16.2 software (SAS Institute Inc, 2021). All other statistical analysis were performed with SPSS (Statistical Package for the Social Sciences) v.18 for Windows (SPSS Inc., Chicago, IL).

Results

Insecticidal tests

Mean mortality percentages of *S. granarius* adults exposed to wheat untreated or treated with different doses of NCZ and DE for 3, 7, 14, and 21 days are reported in Table 1. According to the GLMM, the number of dead insects was significantly correlated with the type of inert dust, the dose applied, and the interaction between these two factors (GLMM post hoc Bonferroni corrected: $P < 0.0001$) (Table 2).

Significant differences (Table 3) in insect mortality among control, DE, and NCZ treatments were observed starting from 7 days of exposure at the lowest dose (0.25 kg/ton) (Figure 1a), and from 3 days for all other doses tested (Figure 1b, c, d, e, f).

In particular, DE showed a mean number of dead insects significantly higher than the control with the lower dose of 0.25 kg/ton and 7 days of exposure (Figure 1a), while with NCZ treatments, a mean number of dead insects significantly higher than control was observed with the 2 kg/ton dose and 14 day-exposure (Figure 1d, e, f).

At all doses tested, no significant differences were found among DE and NCZ (Figure 1). The total insect mortality was reached at the 0.5 kg/ton dose following 14 day-exposure for DE and at 2 kg/ton dose following 21 day-exposure for NCZ (Table 1).

Significant reductions in F1 progeny compared to the control were observed from the dose of 0.5 kg/ton for DE (df: 6, H: 17.362, P : 0.008) and 2 kg/ton for NCZ (df: 6, H: 22.070, P : 0.001) (Table 4). At doses of 0.25 and 0.5 kg/ton, the mean number of emerged adults from DE-treated wheat was significantly lower (0.25 kg/ton U: 0.000, Z: - 2.309, P : 0.01; 0.5 kg/ton U: 0.000, Z: -2.366, P : 0.029) compared to that emerged from NCZ-treated wheat, according to Mann-Whitney U test (Table 4).

Persistence tests

Mean mortality percentages of *S. granarius* after 3, 7, 14, and 21 day-exposure to wheat treated with increasing DE and NCZ doses and infested 3, 6, and 12 months after treatment are reported in Tables 5 - 7. Given that insect mortality for each of the two inert powders remained substantially consistent after 3, 6, and 12 months following grain treatment, the statistical analysis was performed on the mortality data recorded at the longest observation time (Table 8).

Twelve months after wheat treatment with DE, the mean number of dead insects was significantly higher than the control starting from 7 day-exposure to 0.25 and 0.5 kg/ton doses (Figure 2a, b) and from 3 day-exposure at higher doses (Figure 2c, d, e). For NCZ, the mean number of dead insects was significantly higher than control starting from 21 day-exposure to the 2 kg/ton dose (Figure 2d, e). At all doses tested, no significant differences were found between DE and NCZ (Figure 2). Consistent with the results of the insecticidal tests, the total insect mortality was reached at the 0.5 kg/ton dose following 14 day-exposure for DE and at 2 kg/ton dose following 21 day-exposure for NCZ (Table 7).

In insecticidal tests the optimal dose was found to be 1 kg/ton for DE and 2 kg/ton for NCZ. Accordingly, statistical analysis was performed to compare the effectiveness of the treatments at these doses when the infestation occurred immediately after treatment, and 3, 6, or 12 months after (Table 9). The DE effectiveness did not vary significantly when wheat

infestation occurred immediately after treatment or 3, 6, and 12 months later, regardless of the time of exposure (Figure 3a). In the case of NCZ, considering a 3 day-exposure, the insect mortality, with an infestation started 12 months after treatment, was significantly lower than that recorded when wheat was infested immediately after treatment or after 3 months (Figure 3b).

On the other hand, from 7 days of exposure on, no significant differences were observed, irrespective of the interval between treatment application and infestation starting (Figure 3b). According with the Mann-Whitney U test, no significant differences were observed in the F1 progeny emerged from wheat treated with DE and wheat treated with NCZ when infestation occurred 3, 6, or 12 months after treatment, regardless of the dose (Tables 10 - 12).

Discussion

Inert dusts can play an important role within IPM strategies to control stored-product insect pests (Ziaee et al. 2021). Among inert dusts, DEs are apparently the most often tested materials for controlling stored-product pests (Zeni et al. 2021). DEs are the natural silica most distributed worldwide, containing siliceous materials such as aluminum, iron oxide, magnesium, sodium, and lime (Subramanyam and Roesli 2000). One of the main advantages of using inert dusts, in particular DEs, is that they are non-toxic to humans and animals. Indeed, DEs are rated by the Food and Drug Administration as GRAS (Generally Recognized As Safe) for human consumption and are also registered as animal feed additives (Banks and Fields 1995; EFSA 2013 a, b). GRAS compounds comprise zeolites as well, a broad range of microporous, crystalline aluminosilicates of natural or synthetic origin (De Smedt et al. 2015). Some of them have been tested with success against certain stored-product beetle species. However, due to their high variability in composition, the insecticidal activity of each type of zeolite must be carefully investigated before using it to control harmful storage insect pests (Germinara et al. 2019).

In the present study, the insecticidal activity and the persistence of a NCZ was evaluated against *S. granarius* in comparison to a DE. The adult mortality of *S. granarius* maintained on wheat kernels treated with NCZ and DE increased with the dose; however, the NCZ based-treatment reached the 100% adult mortality at slightly higher doses and exposure times compared to DE. Moreover, the mean number of adults emerged from wheat treated with 0.25 and 0.5 kg/ton NCZ doses was significantly higher compared to the same DE

doses. However, starting from 1 and 2 kg/ton doses for DE and NCZ, respectively, the total insect mortality was reached within a short time and the mean progeny per replicate was below one individual. Thus, from a practical point of view, our results suggest that 1 kg/ton for DE and 2 kg/ton for NCZ could be considered the optimal doses to control *S. granarius*. These results, while confirming the effectiveness of DE against *Sitophilus* species (Golob 1997, Korunić 1998, Athanassiou et al. 2005; Kljajić et al. 2010) also highlighted a good insecticidal activity of NCZ towards the granary weevil.

The efficacy of inert dusts depends on different parameters including geographical origin (Vayias and Stephou 2009). Therefore, deepening information on insecticidal activity of zeolites from different geographical origins could be useful to identify the zeolites more effective against stored-product insect pests. In recent years, zeolites of different origins applied directly to grain have been evaluated, alone or in combination with synthetic insecticides, for their insecticidal activity against some of the main stored-product insect pests. Some previous studies reported valuable insecticidal activity of natural zeolites originating from Serbia (Kljajić et al. 2010, Andric et al. 2012). For example, Kljajić et al. (2010) found that natural zeolites were effective in the control of *S. oryzae*, *R. dominica* and *T. castaneum* at doses that did not exceed 1000 ppm. In particular, comparing the effectiveness of two formulations of natural zeolite and a DE, zeolites were as effective as DE against *S. oryzae*, *R. dominica* and *T. castaneum* respectively at 5.0, 3.0 and 2.5 times higher application rates (Kljajić et al. 2010). Moreover, wheat-treatment using a natural zeolite from Egypt in combination with chlorpyrifos-methyl, deltamethrin, or spinosad showed high insecticidal activity against *S. granarius* and *S. oryzae* (Abdelgaleil et al. 2022). Furthermore, the use of natural zeolites on concrete to simulate floor, walls and ceilings of bins and warehouses is worth examining (Karimzadeh et al. 2021). Karimzadeh et al. (2021) tested the insecticidal activity of treatment on concrete surface using a natural zeolite originating from Iran, alone or in combination with pyrethroids, against *Oryzaephilus surinamensis* (L.) and *S. granarius*. *Oryzaephilus surinamensis* was more susceptible to the tested insecticides than *S. granarius*. Applications of two DE formulations and a natural zeolite from Greece were evaluated on concrete and steel surfaces against *S. oryzae*, *R. dominica*, and *T. confusum* (Baliota and Athanassiou 2023). Even at low application rates, the dust formulations tested reached the total adult mortality within one week for all three species. To the best of our knowledge, little is known about the insecticidal activity of natural zeolites originating from Cuba (Germinara et al. 2019).

Long-term protection of stored cereals is one of the most important issue in storage pest management strategy (Vayias et al. 2006). It is known that inert dusts could offer a long-term protection of the grain storage; however, most studies focused on the evaluation of inert dusts in short-term laboratory bioassays, without assessing the persistence of these materials in grain applications (Baliota et al. 2024). In the present study, the persistence of DE and NCZ was evaluated when infestation occurred 3, 6, and 12 months after wheat treatment. The persistence activity of NCZ and DE has been confirmed for up to 12 months from the treatment without significant differences between the two inert dusts. Therefore, given the physical mode of action of inert dusts and the risk of infestation by endophytic-developing insect pests, both inert dusts appear suitable as preventive treatments, ensuring effective grain protection over a considerable storage period.

Our results on DE persistence are consistent with previous studies. In laboratory bioassays, two commercial DE formulations applied on durum wheat and barley were equally effective against *S. oryzae* adults causing high mortality level for about 8 months (Athanassiou et al. 2025). Similarly, two different DE formulations derived from Greece were effective against *S. oryzae* and *R. dominica* when applied on durum wheat and maize for six months (Baliota et al. 2024). The persistence of DE was also studied in semi-fields trials with grain treatments effective against *S. oryzae*, *R. dominica*, and *Trogoderma granatum* Everts for a storage period of 4 months (Rigopoulou et al. 2023). Anyway, little is known on the persistence of natural or synthetic zeolites grain treatments against stored-grain insects (Lü et al. 2017). In a previous study, a natural zeolite from Indonesia applied on maize effectively controlled *S. zeamais* during 3 months of storage (Haryadi et al. 1994). However, this result was obtained using a very high rate (50 kg/ton) that could result in adverse effects on grain physical properties (Andrić et al. 2012). In laboratory bioassays, a synthetic zeolite was effective in controlling *C. maculatus* on concrete surfaces and on treated cowpeas, exhibiting high insecticidal activity for up to 4 months and up to 3 months post-treatment, respectively.

In conclusion, the NCZ tested appears of great interest for practical applications in controlling *S. granarius*. Furthermore, NCZ treatment could ensure a long-term wheat protection of at least one year similar to that provided by DE. However, further large-scale studies are needed to determine the potential application of Cuban zeolites in controlling *S. granarius* in storage facilities.

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Table 1. Percentage mortality (Mean $\pm$ S.E.) of *S. granarius* adults after 3, 7, 14, and 21 days of exposure to wheat treated with different doses of diatomaceous earth (DE) and natural Cuban zeolite (NCZ).

Dose (kg/ton)	Mortality (%)							
	3 days		7 days		14 days		21 days	
	DE	NCZ	DE	NCZ	DE	NCZ	DE	NCZ
0	0	2.5 $\pm$ 1.4	5 $\pm$ 3	2.5 $\pm$ 1.4	6.2 $\pm$ 3.7	3.7 $\pm$ 1.2	7.5 $\pm$ 4.8	7.5 $\pm$ 2.5
0.25	12.5 $\pm$ 7.2	7.5 $\pm$ 5.9	32.5 $\pm$ 5.9	16.2 $\pm$ 4.7	52.5 $\pm$ 7.8	21.2 $\pm$ 5.5	62.5 $\pm$ 7.5	25 $\pm$ 7
0.5	87.5 $\pm$ 2.5	30 $\pm$ 5	92.5 $\pm$ 1.4	41.2 $\pm$ 3.1	100	52.5 $\pm$ 3.2	100	70 $\pm$ 3
1	98.7 $\pm$ 1.2	47.5 $\pm$ 6.3	98.7 $\pm$ 1.2	77.5 $\pm$ 12.5	100	86.2 $\pm$ 10.5	100	92.5 $\pm$ 5.9
2	100	85 $\pm$ 3	100	95 $\pm$ 2	100	98.7 $\pm$ 1.2	100	100
4	98.7 $\pm$ 1.2	95 $\pm$ 2	100	100	100	100	100	100
8	100	96.2 $\pm$ 1.2	100	100	100	100	100	100

Table 2. Generalized Linear Mixed Model (GLMM) with a Binomial distribution performed with inert dust and dose as covariates and total number of dead insects as dependent variable.

Main effects	df	χ^2	P
Inert dust	1	57.7	< 0.0001
Dose	6	3102.5	< 0.0001
Interactions			
Inert dust * Dose	6	71.6	< 0.0001

Table 3. Results of Kruskal-Wallis test to compare the number of dead insects on control wheat and wheat treated with diatomaceous earth (DE) or natural Cuban zeolite (NCZ) in insecticidal tests.

Dose (kg/ton)	Days	Kruskal-Wallis H	<i>P</i>-value
0.25	3	0.407	0.816
	7	8.862	0.012
	14	9.401	0.009
	21	9.163	0.010
0.5	3	10.057	0.007
	7	10.021	0.007
	14	10.353	0.006
	21	10.391	0.006
1	3	10.093	0.006
	7	10.203	0.006
	14	9.609	0.008
	21	9.263	0.010
2	3	10.315	0.006
	7	9.536	0.008
	14	9.540	0.008
	21	10.667	0.005
4	3	8.647	0.013
	7	10.560	0.005
	14	10.667	0.005
	21	10.667	0.005
8	3	9.646	0.008
	7	10.560	0.005
	14	10.667	0.005
	21	10.667	0.005

All Kruskal-Wallis tests were performed with 2 degrees of freedom (df = 2).

Table 4. Total number of F1 progeny (Mean $\pm$ S.E.) of *S. granarius* maintained on wheat treated with different doses of diatomaceous earth (DE) and natural Cuban zeolite (NCZ).

Dose (kg/ton)	DE	NCZ
0	139.5 $\pm$ 7.1 a	146.7 $\pm$ 6.6 a
0.25	13.2 $\pm$ 5.5 ab	54.2 $\pm$ 5.6 ab**
0.5	0.2 $\pm$ 0.2 b	8.2 $\pm$ 1.6 ab**
1	0.7 $\pm$ 0.5 b	7 $\pm$ 2 ab
2	1 $\pm$ 1 b	1.7 $\pm$ 0.5 b
4	1.2 $\pm$ 0.6 b	1.7 $\pm$ 0.8 b
8	0.7 $\pm$ 0.2 b	0.7 $\pm$ 0.2 b

In the same column, values with different letters are significantly different at $P < 0.05$ (Kruskal-Wallis Test followed by Dunn's multiple comparison test with Bonferroni correction).

In the same row, asterisks indicate significant differences ($P < 0.05$) between DE and NCZ at the same dose (Mann-Whitney U test followed by Dunn's comparison test with Bonferroni correction).

Table 5. Percentage mortality (Mean $\pm$ S.E.) of *S. granarius* adults after 3, 7, 14, and 21 days of exposure to wheat treated with different doses of diatomaceous earth (DE) and natural Cuban zeolite (NCZ) when infestation occurred 3 months later.

Mortality (%)								
Dose (kg/ton)	3 days		7 days		14 days		21 days	
	DE	NCZ	DE	NCZ	DE	NCZ	DE	NCZ
0	0	3.3 $\pm$ 1.7	1.7 $\pm$ 1.7	3.3 $\pm$ 1.7	3.3 $\pm$ 1.7	5 $\pm$ 3	5 $\pm$ 3	5 $\pm$ 3
0.25	51.7 $\pm$ 24.6	58.3 $\pm$ 11.7	75 $\pm$ 13	88.3 $\pm$ 7.3	100	100	100	100
0.5	71.7 $\pm$ 6.1	63.3 $\pm$ 1.7	93.3 $\pm$ 4.4	98.3 $\pm$ 1.7	100	100	100	100
1	83.3 $\pm$ 8.3	86.7 $\pm$ 6.7	95 $\pm$ 3	100	100	100	100	100
2	91.7 $\pm$ 6.1	93.3 $\pm$ 4.4	98.3 $\pm$ 1.7	100	100	100	100	100
4	90 $\pm$ 7	95 $\pm$ 2	100	100	100	100	100	100

Table 6. Percentage mortality (Mean $\pm$ S.E.) of *S. granarius* adults after 3, 7, 14, and 21 days of exposure to wheat treated with different doses of diatomaceous earth (DE) and natural Cuban zeolite (NCZ), infestation occurred 6 months later.

Mortality (%)								
Dose	3 days		7 days		14 days		21 days	
(kg/ton)	DE	NCZ	DE	NCZ	DE	NCZ	DE	NCZ
0	0	1.7 $\pm$ 1.7	3.3 $\pm$ 3.3	3.3 $\pm$ 3.3	6.7 $\pm$ 4.4	1.7 $\pm$ 1.7	10 $\pm$ 6	8.3 $\pm$ 4.4
0.25	16.7 $\pm$ 4.4	28.3 $\pm$ 18.3	50 $\pm$ 14	18.3 $\pm$ 3.3	93.3 $\pm$ 3.3	85 $\pm$ 10	100	100
0.5	51.7 $\pm$ 1.7	36.7 $\pm$ 1.7	83.3 $\pm$ 4.4	66.7 $\pm$ 10.1	100	95 $\pm$ 3	100	100
1	75 $\pm$ 3	15 $\pm$ 5	93.3 $\pm$ 3.3	63.3 $\pm$ 7.3	100	93.3 $\pm$ 1.7	100	100
2	55 $\pm$ 10	63.3 $\pm$ 11.7	96.7 $\pm$ 3.3	96.7 $\pm$ 1.7	100	100	100	100
4	93.3 $\pm$ 3.3	78.3 $\pm$ 1.7	100	100	100	100	100	100

Table 7. Percentage mortality (Mean $\pm$ S.E.) of *S. granarius* adults after 3, 7, 14, and 21 days of exposure to wheat treated with different doses of diatomaceous earth (DE) and natural Cuban zeolite (NCZ), infestation occurred 12 months later.

Mortality (%)								
Dose	3 days		7 days		14 days		21 days	
(kg/ton)	DE	NCZ	DE	NCZ	DE	NCZ	DE	NCZ
0	0	0	0	0	8.3 $\pm$ 1.7	5 $\pm$ 5	10 $\pm$ 3	18.3 $\pm$ 10.1
0.25	0	0	45 $\pm$ 5	1.7 $\pm$ 1.7	95	11.7 $\pm$ 1.7	100	60 $\pm$ 18
0.5	6.7 $\pm$ 3.3	1.7 $\pm$ 1.7	85 $\pm$ 3	45 $\pm$ 3	100	90 $\pm$ 3	100	91.7 $\pm$ 1.7
1	76.7 $\pm$ 1.7	0	100	31.7 $\pm$ 9.3	100	76.7 $\pm$ 11.7	100	86.7 $\pm$ 10.9
2	71.7 $\pm$ 1.7	10 $\pm$ 6	100	90 $\pm$ 3	100	96.7 $\pm$ 3.3	100	100
4	65 $\pm$ 3	13 $\pm$ 6	100	86.7 $\pm$ 7.3	100	100	100	100

Table 8. Results of Kruskal-Wallis test to compare the number of dead insects on control wheat and wheat treated with diatomaceous earth (DE) or natural Cuban zeolite (NCZ) in persistence tests.

Dose (kg/ton)	Days	Kruskal-Wallis H	<i>P</i> -value
0.25	3	0	1
	7	6.788	0.034
	14	6.112	0.047
	21	6.713	0.035
0.5	3	3.270	0.195
	7	7.448	0.024
	14	7.513	0.023
	21	7.624	0.022
1	3	7.714	0.021
	7	7.714	0.021
	14	6.889	0.032
	21	6.764	0.034
2	3	6.826	0.033
	7	7.714	0.021
	14	6.788	0.034
	21	6.764	0.022
4	3	7.448	0.024
	7	7.019	0.030
	14	7.714	0.021
	21	6.764	0.022

All Kruskal-Wallis tests were performed with 2 degrees of freedom (df = 2).

Table 9. Results of Kruskal-Wallis test to compare the effectiveness of the treatments at 1 kg/ton for DE and 2 kg/ton for NCZ when the infestation occurred immediately after treatment, and 3, 6, or 12 months thereafter.

Dose (kg/ton)	Days	Kruskal-Wallis H	<i>P</i> -value
1	3	5.549	0.136
	7	4.025	0.259
	14	0	1
	21	0	1
2	3	9.317	0.025
	7	7.244	0.065
	14	3.956	0.266
	21	0	1

All Kruskal-Wallis tests were performed with 2 degrees of freedom (df = 2).

Table 10. Total number of F1 progeny (Mean $\pm$ S.E.) of *S. granarius* maintained on wheat treated with different doses of diatomaceous earth (DE) or natural Cuban zeolite (NCZ) and infested 3 months after treatment.

Dose (kg/ton)	DE	NCZ
0	111.3 $\pm$ 7.3	107.3 $\pm$ 5.8
0.25	5 $\pm$ 3	43.3 $\pm$ 5.9
0.5	2.7 $\pm$ 1.5	17.7 $\pm$ 3.5
1	1.7 $\pm$ 0.3	6.3 $\pm$ 1.8
2	2 $\pm$ 1	2 $\pm$ 1
4	2 $\pm$ 1	1 $\pm$ 1

In the same row, no significant differences ($P < 0.05$) emerged between DE and NCZ at the same dose (Mann-Whitney U test followed by Dunn's comparison test with Bonferroni correction).

Table 11. Total number of F1 progeny (Mean $\pm$ S.E.) of *S. granarius* maintained on wheat treated with different doses of diatomaceous earth (DE) or natural Cuban zeolite (NCZ) and infested 6 months after treatment.

Dose (kg/ton)	DE	NCZ
0	85 $\pm$ 3	86 $\pm$ 4
0.25	5 $\pm$ 1	14 $\pm$ 2
0.5	4 $\pm$ 1	12.7 $\pm$ 1.5
1	1 $\pm$ 1	3.7 $\pm$ 1.2
2	0	0
4	0	0

In the same row, no significant differences ($P < 0.05$) emerged between DE and the natural Cuban zeolite at the same dose (Mann-Whitney U test followed by Dunn's comparison test with Bonferroni correction).

Table 12. Total number of F1 progeny (Mean $\pm$ S.E.) of *S. granarius* maintained on wheat treated with different doses of diatomaceous earth (DE) or natural Cuban zeolite (NCZ) and infested 12 months after treatment.

Dose (kg/ton)	DE	NCZ
0	95 $\pm$ 3	96.7 $\pm$ 3.5
0.25	5 $\pm$ 1	17 $\pm$ 2
0.5	3.3 $\pm$ 1.2	10.3 $\pm$ 1.2
1	1 $\pm$ 1	4 $\pm$ 1
2	0	0
4	0	0

In the same row, no significant differences ($P < 0.05$) emerged between DE and the natural Cuban zeolite at the same dose (Mann-Whitney U test followed by Dunn's comparison test with Bonferroni correction).

11. First insights into the insecticidal mechanism of natural zeolite against *Sitophilus granarius*: effects on the antioxidant defence system and glyoxalase pathway

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Keywords: inert dusts, antioxidant enzymes, glutathione, Glyoxalase I, oxidative stress, dicarbonyl stress.

ABSTRACT

Despite growing interest in natural zeolites as eco-friendly alternatives for stored-grain protection, their insecticidal mechanisms remain poorly understood. Here, we investigated for the first time whether exposure of *Sitophilus granarius* adults to natural Cuban zeolite induces oxidative and dicarbonyl stresses. Mortality and stress biomarkers, including levels of reduced (GSH) and oxidised (GSSG) glutathione as well as activities of antioxidant enzymes (superoxide dismutase, catalase, peroxidases, and glutathione S-transferase) and glyoxalase I (involved in dicarbonyl stress detoxification), were assessed in insects exposed for different times (3, 7, 14 and 21 days) on grain treated with different zeolite doses (0.1, 0.25, 0.5 and 1.0 kg/t).

The main findings showed a marked dose-dependent reduction in the GSH/GSSG ratio (up to -90%) after 7 day-exposure to zeolite, accompanied by increased activities of antioxidant enzymes (up to 2.2-fold for glutathione S-transferase) and glyoxalase I (up to 1.5-fold), suggesting that oxidative and dicarbonyl imbalance may contribute to the insecticidal effect of zeolite. Moreover, these results provide the first evidence in insects of a protective role of glyoxalase I against zeolite-induced dicarbonyl stress. Overall, this study uncovers key

aspects of the insecticidal mode of action of zeolites and support their potential for wider application in sustainable agriculture.

1. INTRODUCTION

During grain storage, attacks of insect pests can result in substantial losses estimated at approximately 10-15% of the global annual production in developed countries [1,2]. The conventional strategies used to manage stored product pests mainly involve chemical insecticides and fumigants, including organophosphates, pyrethroids, neonicotinoids, and phosphine. These chemicals, while effective, are known to cause environmental contamination (soil, water, and air), the development of insect resistance, and negative effects on non-target species, including humans [3–5]. In this context, stored product insect pest management lies in the implementation of integrated pest management programs based on the prevention, monitoring, and the timely application of sustainable control means [6]. Thus, take advantage of all available tools and develop new low-impact control means is fundamental to minimizing losses, production costs, ensuring food quality and safety [7].

The granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), is a major pest of stored cereals, and its ubiquitous distribution and polyphagous nature have prompted the development of effective and environmentally sustainable control strategies [8–10].

Inert dusts, a group of mineral-based powders generally characterized by high physicochemical stability and low toxicity to non-target organisms, have been shown to effectively control several stored-product pests [11–13]. Among these, zeolites, crystalline hydrated aluminosilicates, have been proposed as protectants for stored grains, including *Sitophilus* spp., with recent studies providing further insight into their insecticidal effects [14–16]. The insecticidal activity of inert dusts, including zeolites, is commonly attributed to disruption of the cuticular wax layer and increased cuticular water loss [17,18]. Moreover, their particles can adhere to insect sensory structures, such as sensilla, potentially impairing functions essential for environmental perception and survival [15]. Their application has also been associated with reduced survival and reproductive performance across several stored-product insect species, at rates typically ranging from 500 to 5000 mg/kg [19]. Beyond the direct physical effects exerted on insect cuticular and sensory structures, internal physiological responses, including alterations in oxidative status, have been suggested for synthetic nanoparticles [18], but remain unexplored for natural zeolites.

Oxidative stress is a harmful condition that arises from excessive exposure to adverse environmental conditions (high or low temperatures, UV radiation, ozone, pesticides, etc.), resulting from a disruption of the normal cellular oxidant-antioxidant balance, in favor of oxidant species [20–22]. The main oxidants involved are reactive oxygen species (ROS), which at physiological levels are crucial in regulating several processes as differentiation, apoptosis, immune defence, temperature adaptation, and diapause, whereas when excessively produced, or not efficiently scavenged, result in oxidative damaging of lipids, proteins, and nucleic acids, with the impairment of cellular function and lifespan reduction [20,21]. Thus, also in insects, the coordinated enzymatic and non-enzymatic antioxidant defences appear crucial to maintain their redox homeostasis. In this regard, superoxide dismutase (SOD) converts superoxide radicals into H_2O_2 and O_2 , whereas catalase (CAT) detoxifies H_2O_2 into H_2O and O_2 [20,21]. Peroxidases (PODs), including peroxiredoxins and heme peroxidases, also contribute to H_2O_2 detoxification and are involved in oxidative protection, immune defence, development, and insecticide tolerance [23,24]. Glutathione S-transferases (GSTs) are key enzymes involved in xenobiotic detoxification through conjugation with reduced glutathione (GSH). GSTs also protect against lipid peroxidation by reducing H_2O_2 and lipid hydroperoxides, compensating for the low glutathione peroxidase activity in insects [20,21]. Among non-enzymatic antioxidants, GSH is the most abundant and acts both as a direct ROS scavenger and as a cofactor for antioxidant enzymes. In insects, GSH regeneration from oxidized glutathione (GSSG) is mediated by the thioredoxin system, due to the absence of glutathione reductase [20].

It should be considered that GSH availability also represents a major link between oxidative stress and another harmful condition known as dicarbonyl stress [25]. It is caused by the accumulation of reactive carbonyl species (RCS), the most relevant of which is the α -oxoaldehyde methylglyoxal (MG), by-product of glycolysis and the pentose phosphate pathway [26]. Due to its high reactivity, MG damage cellular macromolecules, leading to advanced glycation and lipoxidation end products (AGEs and ALEs) that impair cell function and may trigger apoptosis [27]. MG detoxification depends on the GSH-dependent glyoxalase system, composed of glyoxalase I (GLYI) and glyoxalase II (GLYII) enzymes. GLYI catalyses the rate-limiting conversion of the MG-GSH hemithioacetal into S-D-lactoylglutathione, subsequently hydrolysed by GLYII to D-lactate, with GSH regeneration [27]. Because of its key role in maintaining low MG levels, GLYI has been widely characterized in many organisms [26,28,29]. In insects, however, information remains scarce and limited to *Drosophila*, used as a human disease model [30,31]. Only Dimov et al. [32]

provided a biochemical characterization of GLYI in *Drosophila*, highlighting differences among species and developmental stages.

The limited knowledge of the mechanisms underlying the insecticidal activity of natural zeolites strongly requires an in-deep analysis of its biochemical aspects, thus the present study aimed to investigate the possible involvement of oxidative and dicarbonyl stress in the toxic effects of a natural Cuban zeolite on adult *S. granarius*. To this aim, key antioxidant biomarkers, including the levels of major redox-related compounds (GSH, GSSG and GSH/GSSG ratio) and the activity of the principal antioxidant enzymes (SOD, CAT, POD, and GST) were assessed, as well as the activity of GLYI, involved in cell protection against dicarbonyl stress. Activity of acetylcholinesterase (AChE), the primary molecular target of organophosphate and carbamate insecticides [33], was also assessed in order to rule out any cholinergic effect for zeolite treatment.

MATERIALS AND METHODS

2.1. Chemicals

All reagents at the highest commercially available purity were purchased from Sigma-Aldrich/Merck KGaA affiliates (Darmstadt, Germany). The Natural Cuban Zeolite (Zeokill, Clinoptilolite-Heulandite 67.5%, Mordenite 32.5%; Bioagrotech s.r.l., Dogana, San Marino Republic) used in the experiments originated from the mechanical processing of a sedimentary rock of volcanic origin formed in Cuba and its chemical composition is 65.65% SiO₂, 11.02% Al₂O₃, 1.07% Na₂O, 1.74 K₂O, 2.68 CaO, 0.93 MgO, 0.09 BaO, 1.60 Fe₂O₃, and 0.26 TiO₂. The 93% of particle size is < 20 µm.

2.2. Insects rearing

A laboratory-reared colony of *S. granarius* (L.) was maintained for six generations at the DAFNE Department of the University of Foggia. Insects were reared following the procedure described by D'Isita et al. [9], using whole kernels of soft wheat (*Triticum aestivum* L., cv. Mec) as the only modification. Prior to use, the kernels were frozen to eliminate any pre-existing infestation, allowed to equilibrate to room temperature, and subsequently placed in cylindrical glass containers (15 cm × 15 cm, Ø x H), covered with a fine metallic mesh (0.5 mm) to ensure ventilation and prevent insect escape. Rearing conditions were maintained at 25 ± 2 °C, 60 ± 5% relative humidity (RH), and in complete darkness. Unsexed adults, 30-day-old, were used for the experiments.

2.3. Experimental design

The bioassays were conducted following the protocols described by Lampiri et al. [34] and D'Isita et al. [35], with minor modifications. Three sets of experiments were carried out using uninfested soft wheat obtained as described in section 2.2.

2.3.1. Mortality experiment (Exp 1)

Samples of 1 kg of soft wheat, either untreated (control) or treated with different zeolite doses - 0.1, 0.25, 0.50, and 1.0 kg zeolite per metric tonne of grain (kg/t), chosen in accordance with previous studies [36,37] and preliminary bioassays - were placed in glass containers (15 x 15 x 30 cm, L x W x H). Each container was manually shaken for 5 min to ensure uniform distribution of the dust. For each replicate, 1000 adults of *S. granarius* were introduced and maintained at 25 ± 2 °C and $60 \pm 5\%$ RH for 3-, 7-, 14-, and 21-day exposure. Three replicates (n=3) were conducted for each treatment and exposure time, resulting in a total of 60 replicates (48 treated and 12 controls). Dead adults were counted after 3, 7, 14 and 21 days of exposure and after this procedure all adults were removed. Mortality was assessed as the percentage of dead individuals relative to the total number of insects in each replicate.

2.3.2. Time-dependent experiment (Exp 2)

To investigate the time-dependent effects of a sublethal dose of zeolite (0.25 kg/t, as obtained in Exp 1) on biochemical markers, 1000 adults of *S. granarius* were reared for 0, 3, 7, 14, and 21 days on 1 kg of either untreated grain (control) or treated with 0.25 kg/t zeolite, following the procedure described for Exp 1.

2.3.3. Dose-dependent experiment (Exp 3)

To investigate the effects of different zeolite doses after a 7-day exposure, 1000 adults of *S. granarius* were reared for 7 days on 1 kg of untreated grain (control) and treated with different doses of zeolite (0.1, 0.25, 0.5, 1.0 kg/t), following the procedure described for Exp 1.

2.4. Biochemical assays

2.4.1. Determination of glutathione pool

Preparation of extracts

After the incubation period, live insects were removed from grain and homogenized using liquid nitrogen. Glutathione extraction was performed following Soccio et al. [29] with minor modifications. Homogenized samples were deproteinized with 5% (w/v) trichloroacetic acid at a 1:5 (w/v, g/mL) ratio for 15 min on ice, and centrifuged at 14500xg for 10 min at 4 °C. Supernatants were neutralized with 0.5 M K-Pi buffer pH 7.0 (1:1.5, v/v). For GSSG determination, 1 mL of neutralized extract was incubated for 30 min with 20 µL of 2-vinylpyridine (VPD) to mask GSH, whereas for total glutathione (GSH and GSSG) measurement a parallel sample was treated with 20 µL of deionized water [38]. Samples were centrifuged at 14500xg for 15 min and supernatants were collected.

Assay of GSH and GSSG contents

Measurements were performed according the assay described by Law et al. [39], involving *i*) the reaction of GSH with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce 5-thio-2-nitrobenzoic acid (TNB) and *ii*) the regeneration of GSH by glutathione reductase (GR) using NADPH. The rate of TNB formation is proportional to GSH concentration. The reaction mixture (0.5 mL) contained 100 mM Na-Pi buffer pH 7.5, 0.2 mM NADPH, 0.6 mM DTNB, and the neutralized supernatants, untreated (2-10 µL) or VPD-treated (5-25 µL). The reaction was initiated by adding 0.5 E.U. of GR and monitored as absorbance increase at 412 nm using a Lambda 45 UV/VIS spectrometer (Perkin Elmer, USA). Total glutathione and GSSG were determined using standard curves prepared with known GSH and GSSG concentrations, respectively. VPD pre-treatment of GSSG standards, as for extracts, improved accuracy by correcting any potential inhibitory effect of residual VPD on GR activity. GSH content was calculated as difference between total glutathione and GSSG.

2.4.2. Determination of enzymatic activities

Preparation of extracts

Live insects removed from grain were homogenized using liquid nitrogen. Enzyme extraction from homogenized samples was performed as described in El-Samad et al. [40], with some modifications, using an ice-cold extraction medium consisting of 50 mM Na-Pi buffer pH 7.20 (1:15 w/v, g/mL). After 30 min of extraction in ice cold bath, the resulting homogenates were centrifuged at 18500xg at 4 °C for 30 min; the supernatants (extracts) were collected and daily used for enzymatic activity assays. For AChE extraction, the medium was supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5%

(v/v) Triton X-100 [41,42], which promotes the release of membrane-bound globular enzyme aggregates.

Determination of protein content

The protein content of extracts was determined following the method of Bradford [43], using a SpectraMax[®] M5 microplate reader. Bovine serum albumin was used as a standard.

Enzymatic activity assays

SOD activity was determined based on its capacity to inhibit pyrogallol autoxidation, according to the protocol described by Marklund and Marklund [44] and modified as in Tiwari et al. [45]. Under alkaline conditions and in the presence of O₂, pyrogallol undergoes autoxidation, which generates superoxide anion and purpurogallin. The assay was performed at 25 °C by monitoring the absorbance increase at 420 nm due to purpurogallin formation using a Lambda 45 spectrometer. The reaction mixture (2 mL) contained 50 mM Tris-HCl buffer pH 8.2 and 1 mM EDTA. The reaction was initiated by the addition of pyrogallol (typically 0.2-0.3 mM), resulting in a control (in the absence of extract) reaction rate - calculated as the highest slope to the experimental curve - equal to $0.020 \pm 0.003 \Delta A_{420 \text{ nm}}/\text{min}$. Measurements were then performed in the presence of the extract obtained as above described, used in the 10-50 μL range. The percentage inhibition of pyrogallol autoxidation induced by the extract was calculated and used to quantify SOD activity, defined as the enzyme amount necessary to achieve a 50% inhibition of pyrogallol autoxidation.

CAT activity was determined based on the method of Aebi [46] with minor modifications, by measuring at 25 °C the decline in absorbance at 240 nm due to H₂O₂ consumption, using a SpectraMax[®] M5 microplate reader (Molecular Devices, USA). The assay mixture (0.2 mL) contained 50 mM Na-Pi buffer pH 7.2, 10 mM H₂O₂ and extract (1-10 μL). The reaction was initiated by the addition of H₂O₂. The reaction rate, calculated as the highest slope to the experimental curve, was used to quantify CAT activity ($\epsilon_{240}=39.4 \text{ mM}^{-1} \cdot \text{cm}^{-1}$).

POD activity was determined as described by Deva et al. [47], with some modifications, by monitoring at 25 °C the absorbance increase at 420 nm due to purpurogallin ($\epsilon_{420}=2.64 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) formation, using a Lambda 45 spectrometer. Unlike the SOD assay, this method relies on the POD-catalyzed oxidation of pyrogallol to purpurogallin in the presence of H₂O₂, under non-alkaline conditions, excluding pyrogallol

autoxidation. The reaction mixture (2 mL) comprised 100 mM K-Pi buffer pH 6.0, 40 mM pyrogallol, 10 mM H₂O₂ and extract (50-200 µL). The reaction was started by the addition of pyrogallol; after 3-4 min, H₂O₂ was added. The rate obtained as difference between the rates calculated after and before addition was used to determine POD activity.

GST activity was measured using the *GST assay Kit* (Sigma-Aldrich) following manufacturer's instructions. The assay was performed by monitoring, at 25 °C and 340 nm, the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH to form the derivative 1-(S-glutathionoyl)-2,4-dinitrobenzene (GS-DNB), using a SpectraMax[®] M5 microplate reader. Extract was assayed in the 0.25-2.0 µL range.

GLYI activity was monitored as described by Schmitz et al. [48] and modified by Soccio et al. [29] and Dimov et al. [32], by following at 240 nm and 25 °C the conversion of hemithioacetal (HA) to S-D-lactoylglutathione (S-LG) ($\epsilon_{240}=2.86 \text{ mM}^{-1} \cdot \text{cm}^{-1}$), using a Lambda 45 spectrometer. HA was preliminarily generated by spontaneous reaction of 8.25 mM MG and 1.05 mM GSH for 30 min in an assay mixture (0.4 mL) consisting of 50 mM Na-Pi buffer pH 7.20. Under these conditions ($K_d=3.0 \text{ mM}$ [48]), HA was 0.75 mM, with residual MG and GSH of 7.5 and 0.3 mM, respectively. The reaction was initiated by adding the extract (5-20 µL).

AChE activity was measured using the *AChE assay Kit* (Sigma-Aldrich) following manufacturer's instructions, using a SpectraMax[®] M5 microplate reader. Thiocholine production resulting from acetylthiocholine hydrolysis was measured at 412 nm, by following the generation of TNB through the reaction of thiocholine with DTNB. Extract was assayed in the 2-10 µL range.

2.5. Data analysis

All data are reported as mean value $\pm$ SD ($n \geq 3$ independent experiments). All statistical analyses were performed by using Statistica software, version 7.0 (StatSoft Inc. 1984-2004, Tulsa, OK 74104, USA). Data of Fig. 1 were transformed to arcsine square-root values. Normal distribution of all data was verified by using the Shapiro-Wilk test. Homogeneity of variances was verified by the Bartlett's test. Data were submitted to a "one-factor" (Figs. 4-5) or "two-factor" (Figs. 1-3) analysis of variance (ANOVA) model. The significant differences were assessed by Duncan's test at 0.05 *P* level of significance. Principal

Component Analysis (PCA) of Fig. 6 was conducted on standardized data, based on the correlation matrix. Score and loading plots were used to interpret variable contributions and sample distribution.

3.RESULTS

3.1 Effect of zeolite dose and exposure time on *S. granarius* mortality (Exp 1)

In this experiment, the effects of different exposure times (3, 7, 14, and 21 days) and zeolite doses (0, 0.1, 0.25, 0.5, and 1.0 kg/t) on adult *S. granarius* mortality were evaluated. Data are presented in **Fig. 1** as a function of either treatment duration (**A**) or zeolite dose (**B**) and in Supplementary Table S1. Under control conditions, mortality progressively increased over time, reaching approximately 40% after 21 days (**Fig. 1A**). A similar time-dependent trend was observed at the lowest zeolite dose (0.1 kg/t) without significant differences with the control at any exposure period. In contrast, 0.25 and 0.5 kg/t zeolite doses substantially enhanced insect mortality, with a sharper dose- and time-dependent increase during the early week of exposure, reaching values at day 7 of about 50% and 60%, respectively (**Fig. 1A**). Beyond 7 days, mortality continued to increase at both doses reaching at day 21 about 75% and 80%, respectively, but at a markedly lower rate than observed during the initial exposure period. The highest zeolite dose (1.0 kg/t) induced a nearly complete insect mortality (90%) after 7 days, with a very limited further increase observed thereafter (**Fig. 1A**).

As shown in **Fig. 1B**, mortality increased with zeolite dose at all exposure times, although the magnitude of the response depended strongly on treatment duration. After 3 days of exposure, mortality exhibited a modest but linear increase with increasing zeolite concentration, reaching about 50% even at the highest dose tested (1.0 kg/t). In contrast, longer exposure periods (7, 14, and 21 days) markedly enhanced zeolite efficacy, with mortality increasing sharply between 0.1 and 0.25 kg/t. Beyond this zeolite dose, mortality continued to increase, although at a substantially lower rate.

3.2 Time-course of glutathione levels and enzyme activities under 0.25 kg/t zeolite exposure (Exp 2)

In this experiment, the levels of GSH and GSSG, as well as the activity of antioxidant enzymes (CAT, SOD, POD, and GST), GLYI and AChE, were evaluated in insects reared on either untreated grain (control) or grain treated with the sublethal zeolite dose of 0.25 kg/t for different exposure periods (0-21 days) (**Figs. 2-3** and Supplementary Tables S2 and S3). As shown in **Fig. 2**, GSH levels declined in both control and zeolite-treated insects during the last week, reaching reductions of about 40-45% relative to initial values by day 21. Concurrently, GSSG approximately doubled by day 21, leading to a decrease of about 70% in the GSH/GSSG ratio in both groups. Notably, treated insects exhibited a significant 20% reduction in GSH content on day 7 compared with controls, accompanied by a marked 40% increase in GSSG levels, leading to a 40% decline in the GSH/GSSG ratio.

As for enzymatic activities, control insects showed only minor changes ($\leq 20\%$) in SOD, CAT, and GST during the first week (**Fig. 3**), while POD and GLYI increased considerably, peaking on day 7 (+50% and +35%, respectively). Subsequently, POD and GLYI returned to baseline levels, CAT remained stable, SOD and GST declined by day 21 (-30% and -35%, respectively). In zeolite-treated insects, all enzymes increased markedly during the first week, peaking on day 7 (from +30% to +80%), while CAT and SOD remained elevated until day 14. Thereafter, activities returned to baseline, except GST, which decreased by about 25% (**Fig. 3**). Interestingly, at each time point, zeolite-treated insects exhibited enzyme activities equal to or higher than the corresponding controls. Once again, the most pronounced increases were observed on day 7 for all enzymes (ranging from about +20% to +40%). For POD, GLYI, and SOD, these increases persisted until day 14 (+25%, +20%, and +20%, respectively) (**Fig. 3**). AChE increased during the first week without significant differences between control and treated insects (**Fig. 3**).

3.3 Glutathione levels and enzyme activities after 7 days of exposure to different zeolite doses (Exp 3)

In this experiment, measurements of glutathione levels and enzymatic activities were carried out after 7 days of rearing insects on untreated grain (control) and treated with increasing doses of zeolite (0.1, 0.25, 0.5, 1.0 kg/t). Results are shown in **Figs. 4-5** as percentages of the control, whereas the corresponding raw data are reported in Supplementary Tables S4-S5.

As shown in **Fig. 4**, zeolite treatment induced a significant dose-dependent reduction in GSH content (ranging from -10% to -70%), with a concomitant marked increase in GSSG level (up to +175%), resulting in a pronounced dose-dependent decline in the GSH/GSSG ratio (up to -90%).

As for enzymes, GST showed a clear dose-dependent response, with activities rising from +25% to +120% compared with the control at zeolite doses between 0.25 and 1.0 kg/t (**Fig. 5**). A similar trend was observed for GLYI, which increased by about +20% to +50% over the same dose range. CAT was found to increase by about 20% at 0.1 kg/t zeolite, and reached approximately +40% at higher doses. Moderate variations were observed for POD (up to +20%) and SOD (up to +15%) at doses above 0.1 kg/t, while AChE remained unchanged (**Fig. 5**).

To identify the traits contributing most to the total variance, PCA analysis performed using all variables measured in Exp. 3. The first two PCA components together explained 96.35% of the cumulative variance (**Fig. 6A**), with PC1 and PC2 accounting for 80.33% and 16.02%, respectively. PC1 was strongly influenced by the GSH/GSSG ratio and the GSH level. All other parameters were negatively associated with PC1, with mortality and GLYI activity showing the strongest negative loadings. PC2 was mainly driven by POD and, to a lesser extent, by SOD and CAT. In contrast, it was negatively associated with AChE and, to a smaller degree, with GST and GSSG content. Correlation analysis revealed a strong positive relationship between GSH content and the GSH/GSSG ratio ($r=0.9845$, $P<0.01$), as well as between GLYI activity and mortality ($r=0.9811$, $P<0.01$). SOD and CAT were also positively correlated ($r=0.9124$, $P<0.05$), as were GSSG content and GST activity ($r=0.9833$, $P<0.01$). In contrast, POD and AChE were not significantly correlated with other variables. The score plot (**Fig. 6B**) shows a clear separation of samples along PC1 according to zeolite doses. Lower zeolite doses cluster on the positive side of PC1, whereas higher doses shift progressively toward the negative side, supporting a zeolite dose-dependent response. On the other hand, separation along PC2 appears less dose-related.

4 DISCUSSION

S. granarius is a major pest of stored cereals, traditionally controlled with synthetic insecticides and fumigants. Concerns about chemical residues, environmental impact, and insect resistance have increased interest in natural zeolites as sustainable, low-toxicity alternatives compatible with Integrated Pest Management programs [14,15,49,50]. Their

insecticidal activity is mainly attributed to physical effects, such as cuticle abrasion causing desiccation and impairment of sensory structures affecting locomotion and reproduction [15,17]. However, the underlying cellular and molecular mechanisms remain poorly understood compared to synthetic nanoparticles [18]. Clarifying these mechanisms would unravel how zeolites affect insect physiology, ultimately supporting their broader application in sustainable grain protection strategies.

Among the physiological processes potentially involved, oxidative stress represents a particularly relevant target. Indeed, several studies have shown that exposure to chemical contaminants and physical stressors can strongly alter ROS production and antioxidant defences in insects [21,22]. Despite this evidence, no information is currently available on natural zeolites. Oxidative stress arises when ROS overwhelm antioxidant defenses, leading to metabolic disturbances and cellular damage [20–22]. The primary enzymatic defense includes SOD, which scavenges superoxide anion, and CAT and PODs, which detoxify H_2O_2 . GSTs contribute to this protective network by using GSH to eliminate hydroperoxides, alongside their role in xenobiotic detoxification. Crucial to redox homeostasis is GSH, acting both as a direct ROS scavenger and as an essential cofactor for antioxidant and detoxification enzymes [21]. GSH is also essential for the catalytic activity of GLYI, the rate-limiting enzyme of the glyoxalase pathway responsible for detoxification of RCS, mainly MG. This function links antioxidant defense with protection against dicarbonyl stress. Accordingly, oxidative and dicarbonyl stress are tightly interconnected processes whose regulation depends on the coordinated action of antioxidant enzymes and the glyoxalase system [25]. However, although GLYI has been investigated in insects, current knowledge is largely restricted to studies on *Drosophila melanogaster* models of human diseases, while its physiological relevance in non-model insects remains poorly understood [30,31]. Within this framework, the present study aimed to explore the biochemical basis of the insecticidal activity of natural zeolites against *S. granarius*, focusing on the potential involvement of oxidative and dicarbonyl stress through an integrated analysis of multiple biochemical markers. Specifically, metabolites having a central role in ROS and MG homeostasis (GSH, GSSG, and the GSH/GSSG ratio) were quantified, together with the activities of key antioxidant enzymes (SOD, CAT, POD, and GST) and of the anti-glycating enzyme GLYI. To study the response of antioxidant and anti-dicarbonyl systems in *S. granarius* exposed to natural Cuban zeolite, two experiments were performed, in which changes dependent on exposure time and zeolite doses were explored, following a preliminary mortality experiment.

As shown in **Fig. 1** (Exp 1), the natural Cuban zeolite, at dose higher than 0.1 kg/t, exhibited effective insecticidal activity against the granary weevil, with a clear dose- and time-dependent effect. In particular, a steep initial increase was observed at 0.25 and 0.5 kg/t during the first week of exposure followed by a more gradual rise over time (Fig. 1A). In contrast, the highest dose (1.0 kg/t) induced a rapid response, with mortality reaching a plateau by day 14, showing that maximum efficacy was achieved earlier. Interestingly, the mortality increased sharply between 0.1 and 0.25 kg/t, after which the rate of increase became more gradual (Fig. 1B). These patterns suggest that lower doses may act more slowly but with progressive accumulation of effects, whereas higher doses induce faster mortality, likely due to increased particle contact and disruption of insect cuticular function [17]. These results are consistent with previous studies on natural zeolites from Serbia, Egypt, and Greece, which reported strong insecticidal activity against *Sitophilus oryzae* and other stored-product beetles within the first 7-14 days of exposure, even at relatively low application rates [11,19,36,37]. Moreover, the dose of 0.25 kg/t produced at 7, 14 and 21 days the largest increment in mortality relative to the previous concentration, indicating a threshold beyond which further increases in zeolite dose (0.50-1.0 kg/t) resulted in progressively smaller gains in mortality.

Based on the results of Exp. 1, the dose of 0.25 kg/t was selected for the biochemical assays in Exp. 2. Besides eliciting the largest incremental increase in mortality (Fig. 1B), this concentration may be regarded as a sublethal threshold, as mortality remained below 70-80% even after 21 days of exposure, ensuring an adequate number of surviving insects for the evaluation over time of antioxidant and anti-glycation defence responses.

As for Exp 2, the results showed a decline in GSH level and a concomitant increase in GSSG content during the last week in control insects, resulting in a 70% reduced GSH/GSSG ratio (**Fig. 2**), consistent with the background mortality observed in control insects (Fig. 1). Given the central role of GSH in redox regulation and MG detoxification, this pattern is consistent with an increase in its consumption to counteract a time-dependent ROS production potentially coupled with onset of dicarbonyl stress [25]. A trend similar to control was observed in insects treated with zeolite at 0.25 kg/t. However, significant differences in GSH (-20%) and GSSG (+40%) levels, as well as in the GSH/GSSG ratio (-40%), were detected compared with controls at day 7. These findings suggest that the physiological time-dependent redox and dicarbonyl imbalance may be exacerbated by zeolite treatment. Notably, this condition appears to reach its peak within the first week, specifically on day 7.

Moreover, results of Exp 2 (**Fig. 3**) showed that that enzyme activities in control insects remained largely stable over time, displaying only moderate fluctuations (CAT). Transient increases (up to +50%) were observed only for POD and GLYI at day 7, whereas SOD and GST declined during the final week. These patterns likely reflect the physiological adjustment of the antioxidant and anti-glycating systems, mainly occurring during the first week, to the stress associated with transfer to new containers [35]. Interestingly, exposure to 0.25 kg/t zeolite dose induced a pronounced activation of all enzymes at each time point, peaking within the first week (day 7), with increases ranging from 30% to 80%. On the contrary, AChE profile showed no significant differences between control and treated insects.

Overall, Exp 2 suggests that sub-lethal zeolite dose does not impact neurotransmission but may induce a mild oxidative and dicarbonyl imbalance, triggering a coordinated antioxidant and anti-glycating response that peaks after one week. Accordingly, day 7 was selected for the Exp 3 as the optimal sampling time for assessing the effects of different zeolite doses. Moreover, insect survival at this time point remained sufficient across all treatments (Fig. 1) to allow the biochemical analyses.

As shown in **Fig. 4**, 7-day zeolite treatment induced a pronounced dose-dependent decrease in GSH levels (10-70% reduction relative to control), alongside with a marked increase in GSSG and a strong reduction in the GSH/GSSG ratio (up to +170% and -90%, respectively, at maximum dose of zeolite), thus providing additional evidence of zeolite-induced redox and dicarbonyl imbalance. Similar GSH depletion has been reported in multiple insect species exposed to heavy metals and pesticides [22,51], as well as to heat stress [21]. Regarding enzymatic activities, Exp 3 (**Fig. 5**) showed a clear dose-dependent response in both antioxidant enzymes and the MG detoxification system. GST and GLYI activities increased significantly, indicating a coordinated activation of GSH-dependent peroxide detoxification and MG scavenging in response to oxidative and dicarbonyl stress. Accordingly, strong positive correlations were observed between GLYI and GST ($r=0.9412$, $P<0.05$). In addition, GSH levels were positively correlated with both GLYI and GST ($r=0.9663$, $P<0.01$ and $r=0.9919$, $P=0.001$, respectively). Consistently, a similarly positive correlation between GLYI and GST was also found in Exp 2 ($r=0.9208$, $P=0.001$), further supporting the functional link between antioxidant and anti-glycating responses. CAT showed a greater increase than POD, suggesting a primary role in H_2O_2 detoxification, while SOD showed only minor modulation, suggesting a limited contribution to ROS

detoxification. In contrast, AChE activity remained unchanged, this making unlikely direct neurotoxic effects for zeolite.

Results of PCA analysis (**Fig. 6A**) identified the GSH/GSSG ratio and the GSH content as the primary contributors to PC1, thus indicating cellular redox status and glutathione homeostasis as the main drivers of overall variability among zeolite treatments. The negative loading of mortality and GSSG content along PC1 suggests that GSH depletion shifts cellular redox balance toward an oxidative and dicarbonyl stress condition, which is associated with increased insect mortality. This metabolic imbalance is also correlated to enhanced activity of the anti-glycating enzyme GLYI and of the detoxification enzyme GST, reflecting a compensatory response to zeolite-induced stress. Conversely, a secondary and considerably smaller proportion of total variance (approximately 16%) is captured by PC2, which is primarily associated with POD, with minor positive contributions from SOD and CAT and a negative loading of AChE. In contrast to PC1, which reflects the dominant antioxidant response, PC2 appears to represent a distinct dimension of physiological regulation of antioxidant enzymes, partially uncoupled from the primary antioxidant pattern. This suggests a secondary modulatory pathway, largely driven by POD and operating independently of cholinergic function, likely contributing to the fine-tuning of antioxidant defences under stress. In addition, **Fig. 6B** indicates that the primary source of variability (PC1) among samples is strongly associated with zeolite dose, while a separation of samples along PC2 appears less strictly dose-driven.

Overall, the PCA and Exp 3 results - consistent with Exp 2 - suggest that zeolite exposure may induce a dose-dependent disruption of glutathione homeostasis, as evidenced by the progressive decline in the GSH/GSSG ratio. This alteration is associated with increased mortality, while neurotransmission remains likely unaffected. This oxidative and dicarbonyl imbalance is also accompanied by a dose-dependent activation of GSH-dependent detoxification mechanisms, specifically GLYI and GST. The other antioxidant enzymes display less dose-sensitive responses, likely representing secondary adaptive responses to zeolite-induced oxidative stress.

Dysregulation of antioxidant defences, manifested by changes in SOD, CAT, POD and GST activities, is widely reported in insects exposed to environmental stressors, such as elevated temperatures [21,52], heavy metals, pesticides, insecticides [22], nanoparticles [53]. These effects are often influenced by exposure dose and duration, as well as species-specific traits, age, social role, and the tissue examined [22]. However, unlike the response observed in the present study in *S. granarius* exposed to natural Cuban zeolite, oxidative imbalance induced

by conventional insecticides and nanoparticles is typically associated with impaired neurotransmission resulting from marked AChE inhibition [54]. Similarly, several plant-derived extracts and essential oils, proposed as eco-friendly alternatives to synthetic insecticides, exert their insecticidal activity through alteration of both AChE and antioxidant enzymes [55–58].

Regarding the mechanisms driving oxidative and dicarbonyl imbalance in *S. granarius* following natural zeolite exposure, dehydration-induced cellular alterations may be hypothesized. Specifically, dehydration due to cuticle abrasion is expected to reduce cellular water potential, increase intracellular ionic strength, and promote cell shrinkage [59–61], thereby compromising membrane integrity and functionality. These alterations can trigger mitochondria dysfunction - a major source of ROS generation - especially by the impairment of electron transport chain and loss of mitochondrial membrane potential [60,62,63], leading to excessive ROS production. The resulting redox homeostasis disruption and glutathione depletion may, in turn, trigger dicarbonyl stress, thus promoting oxidative damage [59,62] and protein carbonylation [25], ultimately contributing to cellular toxicity of zeolite.

However, the mechanistic framework linking zeolite-induced dehydration to mitochondrial dysfunction and oxidative/dicarbonyl stress requires validation through future targeted physiological, biochemical, and molecular studies. Furthermore, investigating different natural zeolite types, insect species, developmental stages, and exposure conditions is important to assess the generality and ecological relevance of these findings. The analysis of additional biomarkers, such as lipid peroxidation products, MG levels, and protein carbonylation, alongside the gene expression of key components of the glyoxalase and antioxidant systems, will provide a more comprehensive picture of the cellular and molecular mechanisms driving insecticidal activity of natural zeolites, ultimately clarifying the contribution of oxidative and dicarbonyl imbalance.

5 CONCLUSIONS

This study provides the first mechanistic evidence of the insecticidal activity of natural zeolite against *S. granarius*. Unlike conventional insecticides, Cuban zeolite toxicity does not involve AChE inhibition, but it is associated to a severe cellular oxidative imbalance, marked by a dose-dependent depletion of the GSH/GSSG ratio, accompanied by a pronounced activation of the detoxification enzyme GST and the ROS-scavenging enzymes CAT, POD and SOD. Importantly, our findings also suggest that dicarbonyl stress may be

central to zeolite toxicity in *S. granarius*, with GLYI activation representing a primary defence against the glycation damage. Overall, these insights expand the limited knowledge of insect glyoxalase pathway and advance understanding of zeolite mode of action, further supporting its potential as a sustainable and eco-friendly alternative for stored-grain protection.

LEGENDS

Fig. 1. Mortality of *S. granarius* adults monitored over a 21-day period following exposure to wheat grain treated with different doses of zeolite. Mortality bioassays were performed, as described in Methods, in insects reared for 3, 7, 14 and 21 days on wheat grains both untreated (control) and treated with different doses of zeolite (0.1, 0.25, 0.5 and 1.0 kg/t). Data are reported as a function of either treatment duration (A) or zeolite dose (B). Data are reported as mean $\pm$ SD ($n \geq 3$). Different letters indicate statistically significant differences at $P \leq 0.05$, according to Duncan's test, following a "two-way" ANOVA model assessing the interactive effects of treatment duration (3, 7, 14, and 21 days) and zeolite dose (0, 0.1, 0.25, 0.5, and 1.0 kg/t).

Fig. 2. Glutathione content in *S. granarius* exposed over a 21-day period to wheat grain treated with 0.25 kg/t zeolite. GSH and GSSG contents were measured, as described in Methods, in extracts obtained from insects reared for 0, 3, 7, 14 and 21 days on untreated (control) and zeolite-treated grain (Exp 2). Data are expressed as nmol/mg tissue and reported as mean $\pm$ SD ($n \geq 3$). The GSH/GSSG ratio was also determined. Different letters indicate statistically significant differences at $P \leq 0.05$, according to Duncan's test, following a "two-way" ANOVA model assessing the interactive effects of treatment duration (0, 3, 7, 14, and 21 days) and zeolite dose (0 and 0.25 kg/t).

Fig. 3. Enzymatic activities in *S. granarius* exposed over a 21-day period to wheat grain treated with 0.25 kg/t zeolite. The enzymatic activities of AChE, POD, GLYI, CAT, GST and SOD were measured, as described in Methods, in extracts obtained from insects reared for 0, 3, 7, 14 and 21 days on untreated (control) and zeolite-treated grain (Exp 2). Data are reported as mean $\pm$ SD ($n \geq 3$). Different letters indicate statistically significant differences at $P \leq 0.05$, according to Duncan's test, following a "two-way" ANOVA model assessing

the interactive effects of treatment duration (0, 3, 7, 14, and 21 days) and zeolite dose (0 and 0.25 kg/t).

Fig. 4. Glutathione content in *S. granarius* after 7 days of exposure to wheat grain treated with different doses of zeolite. The levels in GSH, GSSG and the GSH/GSSG ratio were measured, as described in Methods, in extracts obtained from insects reared for 7 days on untreated (control) and zeolite-treated grain (Exp 3). Data are expressed as a percentage of the control and are presented as mean $\pm$ SD ($n \geq 3$). For each investigated parameter, different letters indicate statistically different values at $P \leq 0.05$, according to Duncan's test, following a "one-way" ANOVA model in which zeolite dose (0, 0.1, 0.25, 0.5, and 1.0 kg/t) was considered as the independent variable. In the control group, GSH and GSSG levels were 0.886 ± 0.051 and 0.135 ± 0.006 nmol/mg tissue, respectively, yielding a GSH/GSSG ratio of 6.57 ± 0.50 .

Fig. 5. Enzymatic activities in *S. granarius* after 7 days of exposure to wheat grain treated with different doses of zeolite. The enzymatic activities (AChE, POD, GLYI, CAT, GST and SOD) were measured, as described in Methods, in extracts obtained from insects reared for 7 days on untreated (control) and zeolite-treated grain (Exp 3). Data are expressed as a percentage of the control and are presented as mean $\pm$ SD ($n \geq 3$). For each investigated parameter, different letters indicate statistically different values at $P \leq 0.05$, according to Duncan's test, following a "one-way" ANOVA model in which zeolite dose (0, 0.1, 0.25, 0.5, and 1.0 kg/t) was considered as the independent variable. Control insects exhibited POD, GLYI, CAT, GST and SOD activities of 0.118 ± 0.008 , 0.186 ± 0.012 , 21.0 ± 0.7 , 0.435 ± 0.050 and 45.56 ± 1.59 EU/mg prot, respectively, while AChE activity was 8.40 ± 0.62 EU/g prot.

Fig. 6. Principal component analysis (PCA) of all measured variables for the five zeolite (ZE) treatments (0, 0.1, 0.25, 0.5 and 1.0 kg/t). (A) Loading plot showing the contribution of each variable to the total variance and the direction of their loadings on the principal components (PC1 and PC2). (B) Score plot showing the distribution of samples according to the ZE treatments.

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Author Contributions

MNL, MS, ID and GSG contributed to the study conception and design. Material preparation, data collection, formal analysis, validation, and data curation were performed by MNL, MS, ID, FL, GP, OMP and GC. Manuscript was written by MNL and MS. MNL, MS, ID, FL, GP and GSG contributed to the review and editing of the manuscript. Funding acquisition was carried out by GSG. Final supervision was provided by MS, MNL and GSG. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interest.

FIGURES AND TABLES

Fig. 1

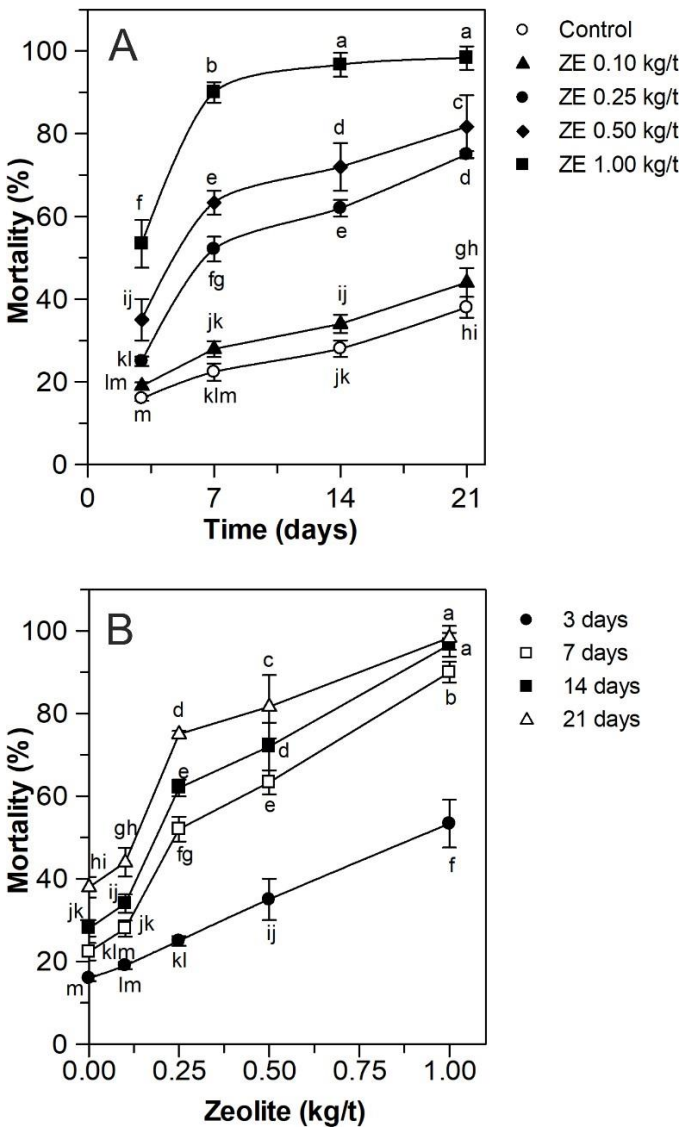


Fig. 2

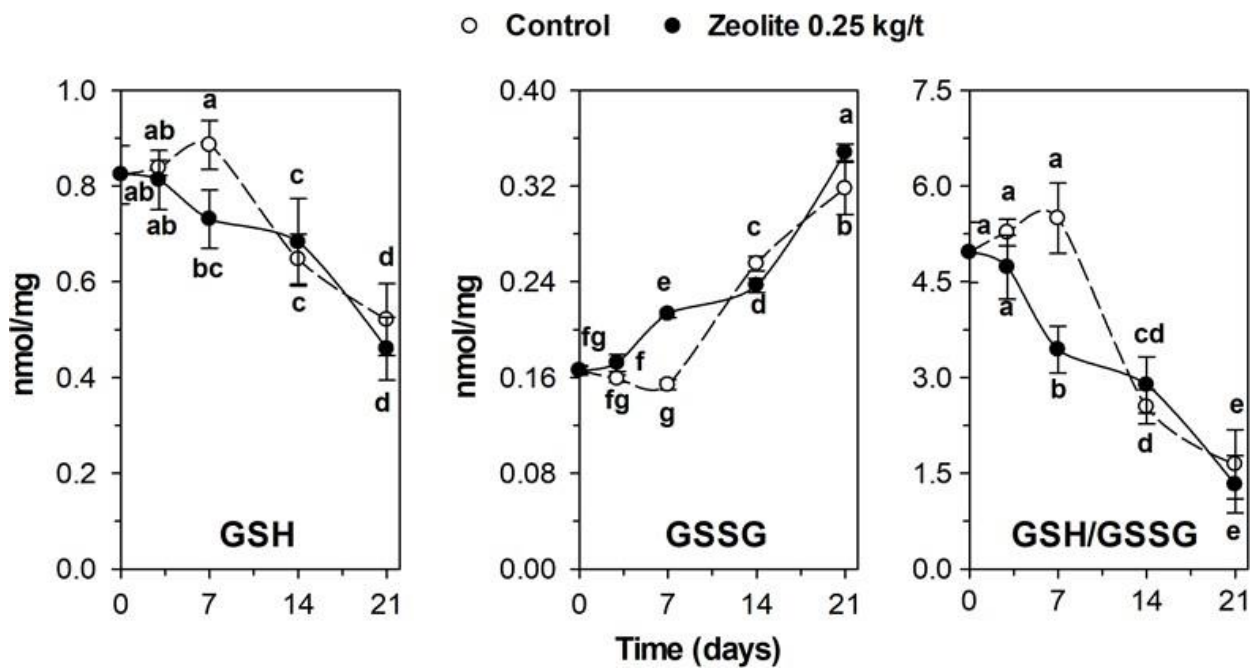


Fig. 3

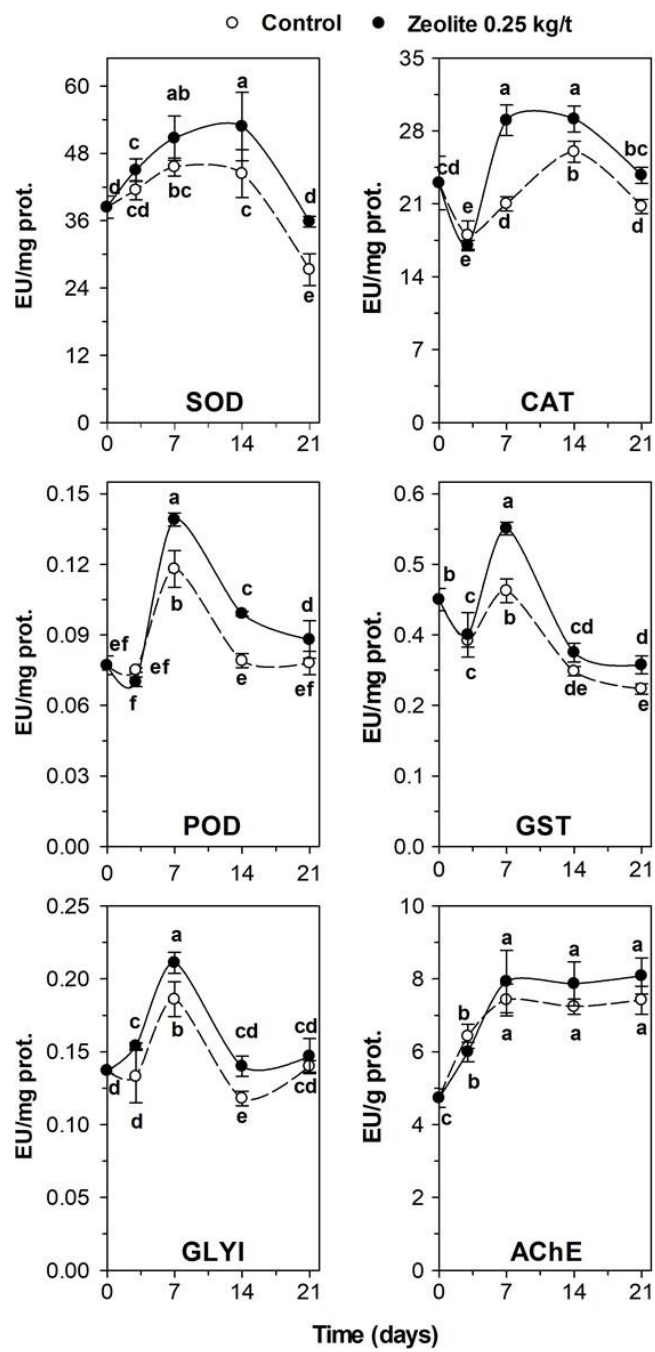


Fig. 4

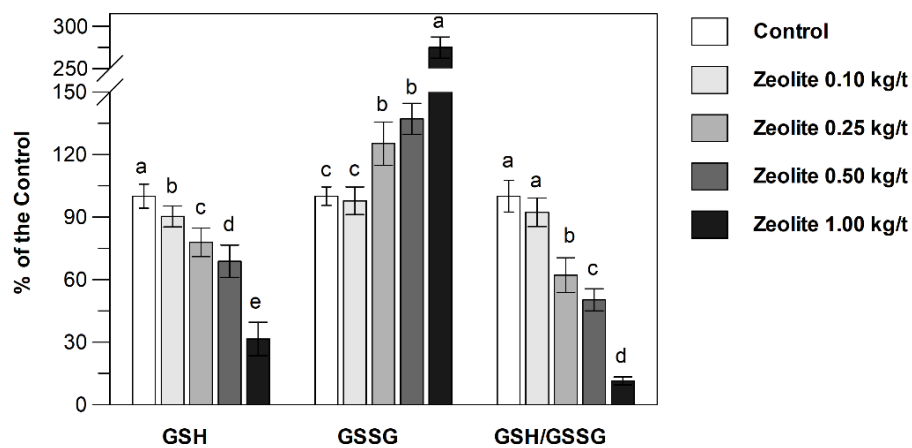


Fig. 5

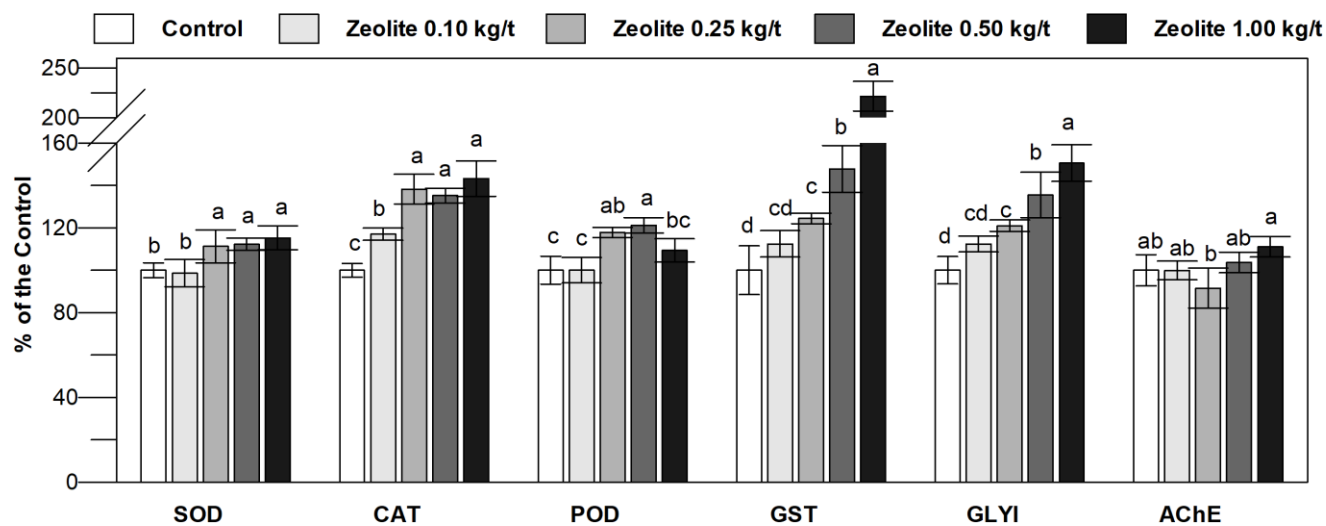
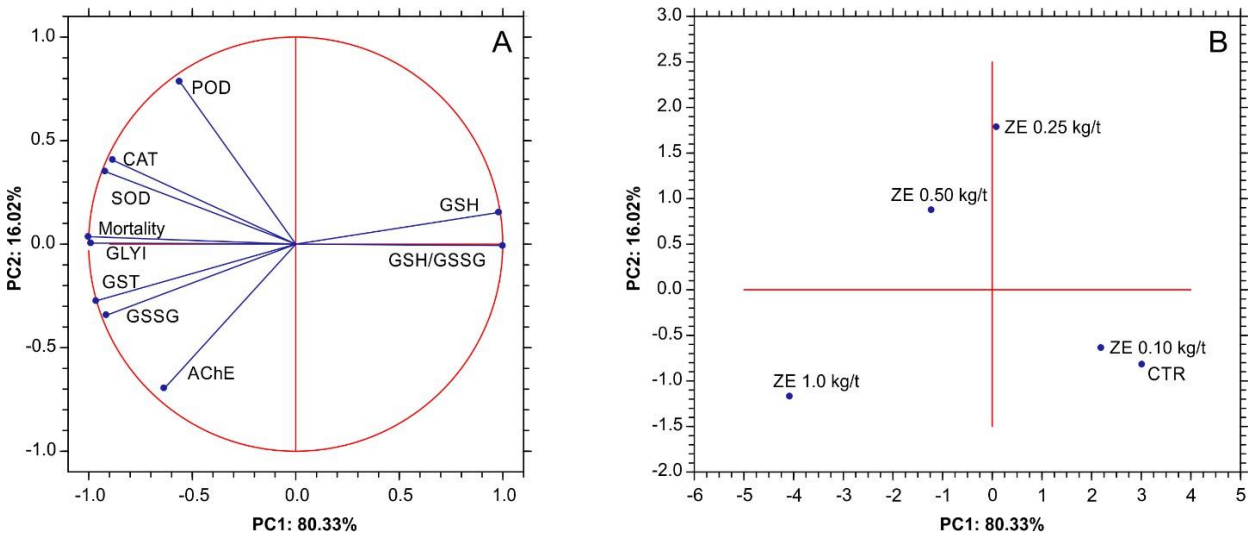


Fig. 6



12. Other studies on stored-product pest management

12.1 Behavioural responses of *Sitophilus granarius* (L.) and *Rhyzopertha dominica* (F.) to odours of old and modern wheat genotypes

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Behavioural responses of *Sitophilus granarius* (L.) and *Rhyzopertha dominica* (F.) to odours of old and modern wheat genotypes

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ABSTRACT

Sitophilus granarius (L.) and *Rhyzopertha dominica* (F.) are among the major stored-product pests worldwide. The identification of low or un-attractive wheat genotypes toward storage pests and their Volatile Organic Compounds (VOCs) profile is useful in breeding programs and in characterization of behaviourally active compounds suitable in developing semiochemical-based control means. Old wheat cultivars are genetic resources useful for breeding purposes. In this study, the VOC profiles of old genotypes, Senatore Cappelli, old Saragolla, Dauno III, and modern wheat varieties, Mec, Ofanto, Svevo, and Faridur, were characterized by headspace - solid phase microextraction (HD-SPME) and gas chromatography coupled with mass spectrometry (GC-MS) and their attractiveness towards *S. granarius* and *R. dominica* adults was evaluated in two-choice pitfall bioassays and Y-tube olfactometer, respectively. *Sitophilus granarius* adults were significantly attracted to odours of all varieties, with females being significantly more attracted by Faridur, Ofanto, Mec, and old Saragolla compared to Svevo. *Rhyzopertha dominica* males and females exhibited a significant olfactory preference for the odours of Faridur, Mec, and old Saragolla. The olfactory preferences of both species could be related to differences emerged in varietal VOC profiles. The most attractive variety for both species, Faridur, was rich in alcohols, alkanes, and terpenes. Old Saragolla, Ofanto, and Mec were rich in alcohols and organic acids. Dauno III was the richest variety in alcohols and lactones, and the poorest in aldehydes and terpenes compared to other varieties. Svevo showed the lowest amounts of alcohols and the highest value of aldehydes; Senatore Cappelli was rich in aldehydes, organic acids, and alkanes. The possible effects of the differences in VOC profiles on the olfactory preferences of insects are discussed. The study offers new knowledge for further investigation testing VOCs of the most and the lowest attractive varieties to identify effective attractants or strong repellents towards *S. granarius* and *R. dominica*.

1. Introduction

Wheat is one of the most important global primary crops essential to humankind with strong consumer's demand and a production of more than 700 thousand tons in 2021 worldwide (Taylor, 1994; Igrejas and Branlard, 2020; FAO, 2023). Insect pests are a serious issue of stored wheat causing postharvest losses of global annual production ranking from around 10% in developed countries to 50% in developing ones, varying according to climatic conditions, storage facilities and pest control measures adopted (Rajendran, 2002; Neethirajan et al., 2007; Fornal et al., 2007; Rajendran, 2020 and references therein; Tadesse, 2020). Stored grains are damaged by different insect pests, including the

granary weevil, *Sitophilus granarius* (L.) (Coleoptera, Curculionidae), and the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera, Bostrichidae). These pests are among the major primary pests of stored cereals leading to quantitative and qualitative losses during storage (Trematerra and Throne, 2012; Majeed et al., 2015; Lemic et al., 2020). Control of storage insects is very difficult due to the increasing legislation limits on the use of pesticides, mainly in developed countries, aimed to reduce environmental and health risks (Handford et al., 2015) and the fast resistance development in target species (Lorini et al., 2007; Song et al., 2011; Boyer et al., 2012; Kocak et al., 2015; Afful et al., 2018; Nayak et al., 2020; Sakka et al., 2022). Furthermore, the early detection of insect infestations in stored grain is essential to apply timely and

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effectively pest management strategies. *Sitophilus granarius* females are able to lay eggs within the grain endosperm (Niewiada et al., 2005). Instead, *R. dominica* females deposit eggs on the surface of kernels or among the frass produced by insects, then the first instar moves into the grains (Edde, 2012). In both species, larvae and pupae develop inside kernels causing hidden grain infestation difficult to be detected (Rotundo et al., 2000; Edde, 2012; Cai et al., 2022a). In this contest, the development of sustainable control tool to manage storage pests is urgently needed (Fields and White, 2002; Germinara et al., 2007; Rajendran and Srikanjini, 2008; Germinara et al., 2012; Adhikary et al., 2014, 2015, 2016; Germinara et al., 2015; Rotundo et al., 2000; Chaudhari et al., 2021; Urrutia et al., 2021).

Semiochemicals are low-molecular-weight signaling chemicals that mediate the interactions between organisms of the same or of different species (Law and Regnier, 1971; Nordlund and Lewis, 1976; Dicke and Sabelis, 1988). Plant Volatile Organic Compounds (VOCs) play a pivotal biological role in plant-insect interactions, since they are the first chemicals detected by insects useful to identify host plants for feeding, mating and oviposition and also to avoid unsuitable habitats and hosts (Dickens, 1984; Visser, 1986; Agelopoulos et al., 1999; Reddy and Guerrero, 2004; Bernays and Chapman, 2007; Piesik et al., 2020). In fact, the granary weevil adults perceive and respond behaviourally (Germinara et al., 2002, 2008) to a wide range of cereal VOCs (Maga, 1978; Zhou et al., 1999) and are attracted to the odours emitted by commercial wheat (Levinson and Kanaujia, 1981; Germinara et al., 2007, 2019). In the same way olfactory cues are important for the lesser grain borer adults to discriminate different kinds of food substrates (Edde and Phillips, 2006; Cao et al., 2024).

The development of semiochemical-based control means toward stored-product pests has showed their possible alternatives to synthetic insecticides (Cox, 2004 and references therein; De Cristofaro et al., 2010; Phillips and Throne, 2010; Abd El-Aziz, 2011; Germinara et al., 2007, 2011, 2012, 2015; Cai et al., 2022b; Cao et al., 2023); thus several studies were aimed to identify, from storage products, chemical volatiles attractants or repellents applicable as direct and indirect control means (Germinara et al., 2012, 2015, 2019, 2019b; Cao et al., 2023, 2024). Furthermore, the use of resistant wheat varieties is considered a preventive agronomic control tools according to the Integrated Pest Management (IPM) approach (Throne et al., 2000; Keba and Sori, 2013); accordingly the identification of wheat varieties, rich in repellent compounds able to modify the insect behaviour, could represent an innovative goal for breeding programs (Germinara et al., 2019).

In recent years, landraces and old cultivars have received a great market interest and thus could represent a source of income for farmers, especially in marginal areas (Perrino, 1993; Stagnari et al., 2008; Ficco et al., 2019; Mefleh et al., 2019). Furthermore, in modern agriculture, growing old wheat genotypes rich in their genetic variability could be a way to safeguard these valuable genetic resources for breeding purposes (Mefleh et al., 2019; D'Isita et al., 2024). To the best of our knowledge, little is known about the behavioural responses of *S. granarius* and *R. dominica* adults to odours emitted by the kernels of old wheat genotypes. In order to identify source of bioactive compounds useful to develop semiochemical-based control tools, the VOC profiles and the olfactory response of three old genotypes (Senatore Cappelli, old Saragolla, Dauno III) and four modern wheat varieties (Mec, Ofanto, Svevo, Faridur) towards the primary stored-cereal pests *S. granarius* and *R. dominica* was here evaluated.

2. Materials and methods

2.1. Insects

Adults of *S. granarius* and *R. dominica* were collected in wheat storage facilities of the Apulia region and reared in the dark at $25 \pm 2^\circ\text{C}$ and $28 \pm 2^\circ\text{C}$, respectively, $60 \pm 5\%$ relative humidity (r.h.) for several generations on durum wheat kernels (var. Simeto) in cylindrical glass

containers ($\varnothing 15 \times 15$ cm) covered with a fine mesh net (0.5 mm). For bioassays, the eighth generation of both species was used.

2.2. Plant materials

The wheat kernel genotypes used in this study, Senatore Cappelli, old Saragolla, Dauno III, Mec, Ofanto, Svevo, and Faridur, were provided by the CREA Research Center for Cereal and Industrial Crops, Italy and grown in a single field trial during 2020–21 at Foggia, Italy ($41^\circ 28' \text{N}$, $119^\circ 15' 32' \text{E}$; 75 m a.s.l.). In particular, the durum wheat kernels were collected from an old landrace Saragolla, two old varieties, released in 1914 (Dauno III) and 1915 (Senatore Cappelli), and three modern varieties, named Ofanto (1990), Svevo (1996), and Faridur (2020). Additionally, the bread wheat variety Mec, released in 1974, was included as a control for its high attractiveness shown in previous studies (Germinara et al., 2019). Faridur derived from an introgression program to transfer the puroindoline genes from hexaploid wheat into durum wheat using Svevo as the recipient variety (Morris et al., 2011; Morris and Fuerst, 2015). Thus, durum wheat varieties Faridur and Svevo shared the same genetic background except for the kernel hardness. The seven genotypes were characterized in a previous study for the level of susceptibility to *S. granarius* and *R. dominica* (D'Isita et al., 2024).

2.3. Two-choice pitfall bioassays

To assess the behavioural response of *S. granarius* adults (3 to 4-week-old) to the kernel odours, two-choice pitfall bioassays were carried out according to previous studies (Phillips et al., 1993; Pike et al., 1994; Germinara et al., 2008).

Briefly, the test arena was a steel container ($\varnothing 30$ cm $\times$ h 7 cm) covered with filter paper (Whatman No. 1) to facilitate insect movements (Pike et al., 1994) and with two diametrically opposed holes ($\varnothing 3$ cm) located 3 cm from the side wall. Glass flasks (500 ml), left empty or containing the stimulus (200 g of uninfested intact kernels of each genotype), were positioned under each hole and the inside surfaces of their necks were coated with mineral oil to prevent captured insects from returning to the arena. Unsexed insects ($n = 20$) deprived of food for at least 4 h were placed under an inverted Petri dish ($\varnothing 3$ cm $\times$ h 1.2 cm) at the center of the test arena and allowed 30 min to acclimate. Then, insects were released for 3 h and given a choice between control (air) and stimulus. During the bioassay, the arena was covered with a steel lid to prevent insects from escaping. Insects were only used once and, at the end of each experiment, the insects that chose the stimulus, the control or those that did not choose any of them were counted and sexed. Experiments were carried out between 09:00 a.m. and 4:00 p.m. in the dark at $25 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ r.h. and 10 replicates for each variety were performed. In each trial, a response index (RI) was calculated by using $\text{RI} = [(T - C)/\text{Tot}] \times 100$, where T is the number of insects responding to the treatment, C is the number of insects responding to the control, and Tot is the total number of insects released (Phillips et al., 1993). Positive and significant RI values indicate attraction to the stimulus, while negative and significant RIs indicate repellence.

2.4. Y-tube olfactometer bioassays

The behavioural response of *R. dominica* adults (3-week-old) to the kernel odours was assessed in a still-air Y-tube olfactometer with a rectangular section (stem 3.5 cm long, each arm 6 cm long at 75°C angle, stem and arms 1 cm wide and 0.7 cm high) in Z-PLA pro material (Zortrax S.A., Olsztyn, Poland), properly designed for this purpose and manufactured using a 3D printer (Zortrax S.A., Olsztyn, Poland). Each arm of the Y-tube terminated with an odour chamber (2 cm long, 2 cm wide, 2 cm high), whose floor was, therefore, 1.3 cm deeper than that of the base and arms. This prevented the insects from perceiving the stimuli. During the experiments, the base and vertical walls of the olfactometer were covered with filter paper (Whatman No. 1) and closed

at the top with a glass plate (18×18 cm). Two-way comparisons were made using uninfested intact kernels (3 g) of each variety as stimulus *versus* control air. Before each experiment, the olfactometer was conditioned for 10 min. After that, individual insects starved for at least 2 h were released at the open end of the Y-tube stem. A choice was recorded when the insect moved 1 cm up an arm of the Y-tube and remained beyond the decision line (marked on both arms) for more than 30 s. The time spent by test insects in each arm was also recorded. After 5 individuals were tested, the filter paper and stimulus were renewed. Treatments and control between arms were switched to avoid position bias. After experiments, the sex of the insects tested was determined by observing their genitalia with a stereomicroscope. For each test stimulus, at least 70 beetles, used only once, were tested. Experiments lasted 10 min and were carried out between 09:00 a.m. and 4:00 p.m. in the dark at 28 ± 2 °C and $60 \pm 5\%$ r.h.

2.5. Volatile organic compounds (VOCs) analysis

The VOCs emitted by the wheat kernels of different varieties tested in behavioural bioassays were identified and quantified using the headspace solid-phase micro-extraction (HS-SPME) technique (Beleggia et al., 2009; Germinara et al., 2019). Wheat kernels were stored at a low temperature (4 ± 1 °C) in glass containers until use. SPME headspace extracts were obtained by exposure for 24 h of SPME fibers (Supelco Co., Bellefonte, PA, USA), coated with either 50/30 μ m of divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS), into the headspace of a 20-mL glass vial containing 3 g of each sample and closed by a cap with a PTFE/Silicon septa (Supelco, Co., Bellefonte, PA). During SPME headspace sampling, the vial was maintained at 30 ± 0.1 °C in a water bath. The fibers were conditioned before use by heating them in the injection port of the gas chromatography (GC) at 270 °C for 30 min, according to the manufacturer's instructions. A fiber cleaning step of 10 min at the conditioning temperature with the split valve opened was performed in the GC injector after every chromatographic run to remove any absorbed residue. Furthermore, before each acquisition, a blank test was made under the same experimental conditions to avoid possible impurities. Each sampling was performed in triplicate. Gas chromatography combined with mass-spectrometry (GC-MS) analyses were performed using an Agilent 7890B series gas chromatograph (Agilent Technologies, Milan, Italy) coupled with an Agilent 5977A mass selective detector (MSD) equipped with an HP-5MS capillary column (30 m $\times$ 0.25 mm ID, 0.5 μ m film thickness, J&W Scientific Inc., Folsom, CA, USA). The VOCs were thermally desorbed at 250 °C for 4 min in splitless mode with a programmed temperature from 40 °C to 250 °C at 5 °C/min and a final holding time of 10 min. Helium was used as the carrier gas at a constant flow rate of 1.25 mL/min. Spectra were recorded in the electron impact mode (ionization energy, 70 eV) in a range of 15–550 amu at 2.9 scans/s. The VOCs identification was reached by comparison of their mass spectra with those of the data system library (NIST11, $p > 90\%$) and, when necessary, by comparing retention times (R.T.) and mass spectra with those of commercially available standards purchased from Sigma-Aldrich Inc. (Milan, Italy). A mixture of a continuous series of straight-chain hydrocarbons, C5–C40 (Alkane Standard Solution C5–C40, Sigma Aldrich, Milan, Italy), was injected into an HP-5MS column under the same conditions previously described to obtain the Linear Retention Indices (RIs) (Van Den Dool and Kratz, 1963). The relative abundance of each compound was calculated using the integrated peak area data from the GC-MS trace.

2.6. Statistical analysis

Data were submitted to Shapiro-Wilk's test, Levene's test, and then to the analysis of variances (ANOVA) followed by Tukey's HSD test for mean comparisons.

In the two-choice pitfall bioassays, the significance of the mean RIs in each treatment was evaluated by the Student's *t*-test for paired

comparisons (Phillips et al., 1993). Furthermore, the number of males and females was analyzed by paired sample *t*-test and for each experiment, the sex ratio percentage was calculated. In Y-tube olfactometer bioassays, the significance of differences between the number of *R. dominica* adults choosing the treatment or control arm of the olfactometer were compared using χ^2 test. The differences between the time spent by insects in each arm were analyzed by paired sample *t*-test. Statistical analyses were performed with SPSS (Statistical Package for the Social Sciences) v.18 for Windows (SPSS Inc., Chicago, IL).

The unsupervised method of Hierarchical Cluster Analysis (HCA), useful to group various samples based on their similarity without knowledge about the number of clusters to be established (Tufariello et al., 2023), was run with the aim of grouping the varieties tested in dependence of chemical classes identified in the VOCs profile and the mean percentages of VOCs GC peak areas.

3. Results

3.1. Behavioural response of *S. granarius* in the two-choice pitfall bioassays

The mean sex ratio percentage was in a range between 49.1 ± 2.0 for Mec and 54.5 ± 4.0 for Svevo, without significant differences in the number of males and females of insects tested (Tables 1 and 2). The granary weevil adults showed positive and significant ($P < 0.001$; Student's *t*-test) RIs that indicate actual attraction in response to the odours of all genotypes studied (Tables 1 and 2). The RI registered for *S. granarius* males ranged between 50.71 ± 10.1 (Dauno III) and 77.91 ± 8.0 (Faridur), without significant differences ($F = 1.939$; $df = 6$; $P = 0.090$) among varieties tested (Table 1). Instead, for females the RI ranged between 34.02 ± 9.1 (Svevo) and 77.55 ± 5.5 (Faridur). The RI of Svevo (34.0 ± 9.1) was significantly lower compared to those calculated for old Saragolla (68.73 ± 4.1), Mec (71.87 ± 5.8), Ofanto (73.43 ± 8.3) and Faridur (77.55 ± 5.5) (Table 2).

3.2. Behavioural response of *R. dominica* in Y-tube olfactometer bioassays

In Y-tube behavioural bioassays, *R. dominica* males and females presented with intact kernels vs. clean-air control exhibited significant preference for Faridur (male: $\chi^2 = 7.111$, $df = 1$, $P = 0.008$; female: $\chi^2 = 5.121$, $df = 1$, $P = 0.024$), Mec (male: $\chi^2 = 3.846$, $df = 1$, $P = 0.050$; female: $\chi^2 = 5.233$, $df = 1$, $P = 0.022$), and old Saragolla (male: $\chi^2 = 5.121$, $df = 1$, $P = 0.024$; female: $\chi^2 = 54.172$, $df = 1$, $P = 0.041$). In addition, for the same varieties, both sexes also spent significantly more time in the treatment arm (Tables 3 and 4). On the other hand, odours from the modern varieties Ofanto and Svevo and the old varieties Senatore Cappelli and Dauno III did not elicit a significant attraction in *R. dominica* adults evaluated either as first choice and time spent in the treatment arm when clean air was the alternative.

3.3. Volatile organic compounds (VOCs) analysis

A total of 27 VOCs were identified in the headspace of the varieties studied including: 9 alcohols, 7 aldehydes, 4 alkanes, 2 terpenes, 1 organic acid, 1 benzene derivative, 1 lactone, 1 ketone, and 1 other compound. The percentages of the compounds detected, expressed as relative abundance, are reported in Table 5.

According to the HCA run to group the varieties studied in dependence on chemical classes, two clusters were observed (Fig. 1). The first cluster included Senatore Cappelli and Svevo together with Faridur and Mec, the second one contained Ofanto and old Saragolla plus Dauno III (Fig. 1). The HCA run to cluster varieties according to the mean percentages of VOCs GC peak areas showed two clusters (Fig. 2). The first one consisted of old Saragolla and Ofanto plus Mec and Dauno III and the second one contained Faridur and Svevo together with Senatore

Table 1

Behavioural responses in two-choice pitfall bioassays of *S. granarius* males to odours emitted by kernels (200 g) of modern wheat (Mec, Ofanto, Svevo, Faridur) and old wheat (Dauno III, old Saragolla, Senatore Cappelli) varieties vs. control air.

Genotype	Males (Mean $\pm$ S.E.)	Kernel odours (Mean $\pm$ S.E.)	Control air (Mean $\pm$ S.E.)	Student's <i>t</i> -test		Response index (Mean $\pm$ S.E.)
				<i>t</i> value	<i>P</i> value	
<i>Bread wheat</i>						
Mec	9.25 $\pm$ 0.4 a	6.86 $\pm$ 0.5	0.43 $\pm$ 0.2	10.510	<0.001	70.37 $\pm$ 7.01 a
<i>Durum wheat</i>						
Ofanto	9.22 $\pm$ 0.6 a	7.67 $\pm$ 0.6	1.00 $\pm$ 0.2	9.177	<0.001	71.43 $\pm$ 5.4 a
Svevo	11.10 $\pm$ 0.8 a	8.70 $\pm$ 0.8	2.30 $\pm$ 0.4	6.532	<0.001	57.52 $\pm$ 7.01 a
Faridur	10.50 $\pm$ 0.5 a	9.10 $\pm$ 0.5	1.00 $\pm$ 0.4	9.847	<0.001	77.91 $\pm$ 8.02 a
Dauno III	10.56 $\pm$ 0.6 a	7.56 $\pm$ 0.6	2.44 $\pm$ 0.5	5.303	0.001	50.71 $\pm$ 10.1 a
Old Saragolla	10.20 $\pm$ 0.6 a	7.70 $\pm$ 0.5	1.70 $\pm$ 0.4	11.163	<0.001	61.36 $\pm$ 6.8 a
Senatore Cappelli	10.67 $\pm$ 0.8 a	7.89 $\pm$ 0.6	2.56 $\pm$ 0.4	7.761	<0.001	51.35 $\pm$ 7.6 a

In the same column, values with different letters are significantly different at $P < 0.05$.

Table 2

Behavioural responses of *S. granarius* females to odours emitted by kernels (200 g) of modern wheat (Mec, Ofanto, Svevo, Faridur) and old wheat (Dauno III, old Saragolla, Senatore Cappelli) varieties, in two-choice pitfall bioassays.

Genotype	Females (Mean $\pm$ S.E.)	Kernel odours (Mean $\pm$ S.E.)	Control air (Mean $\pm$ S.E.)	Student's <i>t</i> -test		Response index (Mean $\pm$ S.E.)
				<i>t</i> value	<i>P</i> value	
<i>Bread wheat</i>						
Mec	9.63 $\pm$ 0.5 a	7.14 $\pm$ 0.3	0.80 $\pm$ 0.2	8.000	<0.001	71.87 $\pm$ 5.8 b
<i>Durum wheat</i>						
Ofanto	8.78 $\pm$ 0.4 a	7.33 $\pm$ 0.4	1.00 $\pm$ 0.4	9.216	<0.001	73.43 $\pm$ 8.3 b
Svevo	9.30 $\pm$ 0.8 a	6.10 $\pm$ 0.6	3.20 $\pm$ 0.5	3.934	0.003	34.02 $\pm$ 9.1 a
Faridur	9.50 $\pm$ 0.4 a	8.40 $\pm$ 0.5	1.00 $\pm$ 0.3	11.045	<0.001	77.55 $\pm$ 5.5 b
Dauno III	9.11 $\pm$ 0.5 a	7.22 $\pm$ 0.6	1.78 $\pm$ 0.4	6.519	<0.001	59.69 $\pm$ 9.01 ab
Old Saragolla	8.90 $\pm$ 0.6 a	7.10 $\pm$ 0.5	1.00 $\pm$ 0.2	11.597	<0.001	68.73 $\pm$ 4.1 b
Senatore Cappelli	9.33 $\pm$ 0.7 a	7.44 $\pm$ 0.4	1.78 $\pm$ 0.4	10.251	<0.001	63.22 $\pm$ 7.5 ab

In the same column, values with different letters are significantly different at $P < 0.05$.

Table 3

Response of *R. dominica* males in a Y-tube olfactometer to different odour sources emitted by kernels (3 g) of modern wheat (Mec, Ofanto, Svevo, Faridur) and old wheat (Dauno III, old Saragolla, Senatore Cappelli) varieties.

Genotype	N ^a	First choice (Mean)			Number of minutes spent in arm (mean ± S.E.)			
		Treated ^b	X ²	P value	Treated	Control	t value	P value
<i>Bread wheat</i>								
Mec	26(26)	0.69	3.846	0.050	5.01 ± 0.7	1.77 ± 0.6	2.569	0.017
<i>Durum wheat</i>								
Ofanto	32(29)	0.55	0.310	0.577	2.97 ± 0.6	2.94 ± 0.7	0.027	0.979
Svevo	39(39)	0.62	2.077	0.150	4.06 ± 0.6	2.69 ± 0.6	1.202	0.237
Faridur	36(36)	0.72	7.111	0.008	5.09 ± 0.6	2.22 ± 0.6	2.397	0.022
Dauno III	47(46)	0.50	0	1.000	3.67 ± 0.6	3.28 ± 0.6	0.362	0.719
Old Saragolla	36(33)	0.70	5.121	0.024	3.97 ± 0.6	1.65 ± 0.5	2.350	0.025
Senatore Cappelli	38(37)	0.46	0.243	0.622	3.46 ± 0.1	3.43 ± 0.1	0.028	0.977

^a Total sample size (*N*, number of individuals that made a choice in parentheses).

^b Proportion of individuals (of those that made a choice) that chose the treated arm first.

Table 4

Response of *R. dominica* female in a Y-tube olfactometer to different odour sources emitted by kernels (3 g) of modern wheat (Mec, Ofanto, Svevo, Faridur) and old wheat (Dauno III, old Saragolla, Senatore Cappelli) varieties.

Genotype	N ^a	First choice (Mean)			Number of minutes spent in arm (mean ± S.E.)			
		Treated ^b	X ²	P value	Treated	Control	t value	P value
<i>Bread wheat</i>								
Mec	44(43)	0.67	5.233	0.022	4.31 ± 0.5	2.04 ± 0.5	2.348	0.024
<i>Durum wheat</i>								
Ofanto	38(33)	0.58	0.758	0.384	3.56 ± 0.6	2.63 ± 0.6	0.827	0.414
Svevo	31(29)	0.52	0.034	0.853	3.59 ± 0.7	3.29 ± 0.7	0.221	0.827
Faridur	34(33)	0.70	5.121	0.024	4.36 ± 0.6	1.74 ± 0.5	2.461	0.019
Dauno III	23(22)	0.59	0.727	0.394	3.74 ± 0.8	2.32 ± 0.7	1.050	0.305
Old Saragolla	34(29)	0.69	4.172	0.041	4.15 ± 0.6	1.62 ± 0.5	2.249	0.021
Senatore Cappelli	32(29)	0.48	0.034	0.853	2.94 ± 0.6	3.44 ± 0.7	−0.397	0.694

^a Total sample size (*N*, number of individuals that made a choice in parentheses).

^b Proportion of individuals (of those that made a choice) that chose the treated arm first.

Table 5

VOCs levels detected in the head-space of modern wheat (Mec, Ofanto, Svevo, Faridur) and old wheat (Dauno III, old Saragolla, Senatore Cappelli) varieties.

Compounds	R.T.	RI Lit ^a	RI Exp ^b	Area (%) (Mean ± S.E.)						
				Mec	Ofanto	Svevo	Faridur	Senatore Cappelli	Old Saragolla	Dauno III
<i>Alcohols</i>										
1-Pentanol	4.79	766	765	0.9 ± 0.1	1.0 ± 0.1	2.8 ± 0.1	2.9 ± 0.2	1.3 ± 0.01	1.7 ± 0.2	1.1 ± 0.1
2,3-Butanediol	5.08	802	802	1.6 ± 0.2	4.3 ± 0.3	2.7 ± 0.4	2.0 ± 0.2	2.5 ± 0.5	9.4 ± 0.5	16.2 ± 2.5
1-Hexanol	7.16	867	867	1.0 ± 0.2	1.7 ± 0.2	2.4 ± 0.3	3.8 ± 1.2	1.3 ± 0.1	1.6 ± 0.2	1.8 ± 0.3
1-Heptanol	10.16	969	943	0.4 ± 0.0	0.5 ± 0.1	0.9 ± 0.02	0.8 ± 0.1	0.5 ± 0.0	0.5 ± 0.1	–
1-Hexanol, 2-ethyl-	11.99	1015	999	10.1 ± 1.9	5.7 ± 0.6	4.1 ± 0.1	4.5 ± 0.1	4.7 ± 0.3	3.9 ± 0.6	3.4 ± 0.2
Benzyl Alcohol	12.20	1036	1005	6.1 ± 0.4	6.8 ± 0.2	1.7 ± 0.2	3.0 ± 0.3	4.4 ± 1.0	3.8 ± 0.5	10.9 ± 1.0
1-Octanol	13.27	1038	1039	1.3 ± 0.1	1.1 ± 0.1	1.5 ± 0.1	1.5 ± 0.0	1.1 ± 0.1	1.2 ± 0.1	0.4 ± 0.0
Phenylethyl Alcohol	14.65	1109	1082	3.3 ± 0.2	3.9 ± 0.2	1.0 ± 0.01	1.7 ± 0.1	1.8 ± 0.5	3.3 ± 0.5	3.1 ± 0.4
1-Nonanol	16.26	1169	1134	1.5 ± 0.1	1.8 ± 0.1	1.3 ± 0.1	2.2 ± 0.01	1.5 ± 0.2	2.3 ± 0.3	1.0 ± 0.01
Total alcohols				26.2 ± 1.8	27.0 ± 0.5	18.4 ± 0.1	22.4 ± 0.2	19.1 ± 1.8	27.7 ± 1.5	37.9 ± 1.7
<i>Aldehydes</i>										
Hexanal	5.47	806	803	3.7 ± 0.9	4.4 ± 0.6	18.5 ± 1.0	8.8 ± 1.0	10.0 ± 0.5	8.2 ± 1.0	1.7 ± 0.3
Heptanal	8.11	894	901	0.8 ± 0.1	0.4 ± 0.04	0.9 ± 0.03	0.6 ± 0.1	0.7 ± 0.02	0.8 ± 0.1	–
Benzaldehyde	9.97	928	937	4.4 ± 0.4	4.3 ± 0.3	1.5 ± 0.1	2.9 ± 0.2	3.5 ± 0.9	1.7 ± 0.4	5.5 ± 0.2
Octanal	11.22	1002	975	0.9 ± 0.1	0.4 ± 0.04	0.8 ± 0.03	0.6 ± 0.02	0.5 ± 0.01	0.5 ± 0.1	–
Nonanal	14.31	1089	1071	3.9 ± 0.6	1.5 ± 0.04	3.5 ± 0.3	2.2 ± 0.4	1.7 ± 0.2	1.9 ± 0.6	1.8 ± 0.5
Decanal	17.27	1207	1168	4.1 ± 0.4	2.2 ± 0.3	3.3 ± 0.2	2.3 ± 0.5	3.4 ± 0.2	4.1 ± 1.0	0.8 ± 0.1
Dodecanal	22.66	1405	1364	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.3 ± 0.01	2.9 ± 0.1	0.8 ± 0.2	0.4 ± 0.0
Total aldehydes				18.7 ± 0.2	14.0 ± 1.1	29.6 ± 0.3	17.7 ± 0.3	22.6 ± 1.6	18.0 ± 0.7	10.2 ± 0.2
<i>Alkanes</i>										
Tridecane	19.82	1300	1256	2.1 ± 0.3	3.3 ± 0.2	2.3 ± 0.1	3.3 ± 0.1	3.5 ± 0.6	1.2 ± 0.1	1.1 ± 0.1
Tetradecane	22.40	1400	1354	2.7 ± 0.3	3.6 ± 0.6	14.7 ± 1.2	18.4 ± 2.2	11.3 ± 0.8	2.4 ± 0.01	2.9 ± 0.2
Dodecane	17.07	1200	1161	1.2 ± 0.2	0.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	5.1 ± 0.9	0.5 ± 0.04	1.5 ± 0.1
Octadecane	31.40	1794	1745	1.4 ± 0.1	0.9 ± 0.2	–	0.1 ± 0.01	0.4 ± 0.1	1.5 ± 0.8	–
Total alkanes				7.4 ± 0.5	8.6 ± 1.0	18.3 ± 0.5	23.1 ± 1.1	20.4 ± 2.3	5.6 ± 0.9	5.5 ± 0.1
<i>Terpenes</i>										
Limonene	12.06	1030	1001	3.6 ± 0.5	0.8 ± 0.05	2.5 ± 0.2	8.2 ± 0.5	1.8 ± 0.2	1.5 ± 0.2	0.6 ± 0.01
Isobornyl acetate	19.65	1280	1250	0.6 ± 0.1	0.4 ± 0.1	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01	0.3 ± 0.02	0.3 ± 0.01
Total terpenes				4.3 ± 0.5	1.2 ± 0.1	2.6 ± 0.1	8.4 ± 0.5	1.9 ± 0.2	1.8 ± 0.2	0.9 ± 0.01
<i>Organic acids</i>										
Acetic Acid	2.89	633	623	19.4 ± 4.1	32.5 ± 2.7	22.4 ± 2.5	17.3 ± 3.5	25.3 ± 2.4	33.9 ± 1.4	21.9 ± 2.0
Total organic acids				19.4 ± 4.1	32.5 ± 2.7	22.4 ± 2.5	17.3 ± 3.5	25.3 ± 2.4	33.9 ± 1.4	21.9 ± 2.0
<i>Benzene derivatives</i>										
p-Xylene	7.27	888	881	11.8 ± 1.9	1.2 ± 0.2	1.3 ± 0.1	2.0 ± 0.5	1.8 ± 0.2	2.1 ± 0.3	1.4 ± 0.1
Total benzene derivatives				11.8 ± 1.9	1.2 ± 0.2	1.3 ± 0.1	2.0 ± 0.5	1.8 ± 0.2	2.1 ± 0.3	1.4 ± 0.1
<i>Lactones</i>										
Butyrolactone	8.52	915	917	3.1 ± 0.8	8.2 ± 0.7	4.3 ± 0.2	5.0 ± 0.1	4.2 ± 0.1	5.5 ± 1.0	17.4 ± 0.5
Total lactones				3.1 ± 0.8	8.2 ± 0.7	4.3 ± 0.2	5.0 ± 0.1	4.2 ± 0.1	5.5 ± 1.0	17.4 ± 0.5
<i>Ketones</i>										
5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	23.77	1421	1408	1.3 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	1.5 ± 0.1	1.2 ± 0.03	1.0 ± 0.05	1.4 ± 0.1
Total ketones				1.3 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	1.5 ± 0.1	1.2 ± 0.03	1.0 ± 0.05	1.4 ± 0.1
<i>Others</i>										
Phenol	10.49	995	953	7.9 ± 0.8	6.4 ± 0.3	2.4 ± 0.5	2.6 ± 0.3	3.5 ± 1.1	4.2 ± 0.5	3.5 ± 0.1
Total others				7.9 ± 0.8	6.4 ± 0.3	2.4 ± 0.5	2.6 ± 0.3	3.5 ± 1.1	4.2 ± 0.5	3.5 ± 0.1

^a RI_{Lit} = linear retention index from the literature.

^b RI_{exp} = determined linear retention index against mixture of n-alkanes (C5–C40) on HP-5MS column.

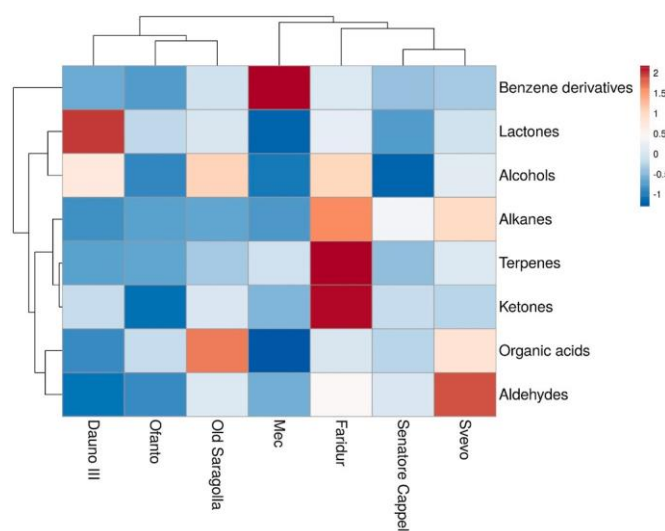


Fig. 1. Dendrogram of Hierarchical Cluster Analysis (HCA) run to group the varieties tested in dependence of chemical classes identified in the VOCs profile.

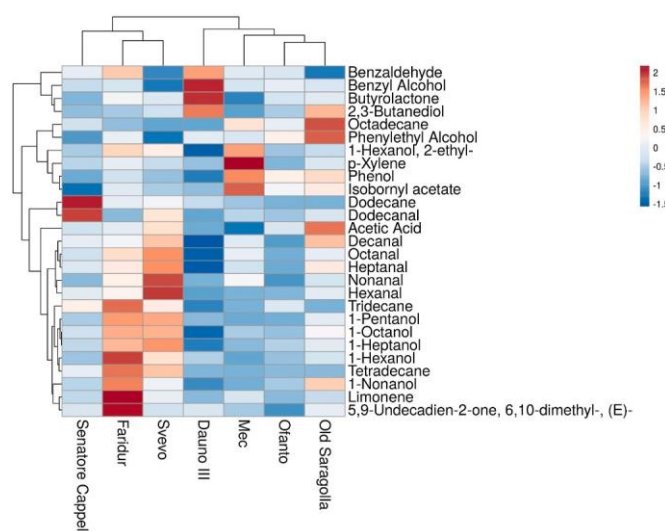


Fig. 2. Dendrogram of Hierarchical Cluster Analysis (HCA) run to group the varieties tested in dependence of the mean percentages of VOCs GC peak areas.

Cappelli (Fig. 2). In particular, in Dauno III VOCs profile the highest values of alcohols (37.9 ± 1.7) and lactones (17.4 ± 0.5), and the lowest values of aldehydes (10.2 ± 0.2) and terpenes (0.9 ± 0), compared to the other varieties, were recorded. The most abundant alcohols were 2,3-butanediol (16.2 ± 2.5) and benzyl alcohol (10.9 ± 1.0), whilst, lactones were represented only by butyrolactone (17.4 ± 0.5).

The most represented chemical classes, in Faridur VOCs profile, were alkanes (23.1 ± 1.1) and alcohols (22.4 ± 0.2). Besides, compared to the other varieties studied, Faridur showed the highest presence of alkanes and terpenes (8.4 ± 0.5) (Table 5). The most abundant alcohols were 1-hexanol, 2-ethyl- (4.5 ± 0.1), 1-hexanol (3.8 ± 1.2), benzyl alcohol (3.0 ± 0.3), and 1-pentanol (2.9 ± 0.2), while tetradecane (18.4 ± 2.2) and limonene (8.2 ± 0.5) were the most abundant alkane and terpene, respectively. By contrast, in Svevo VOCs profile the lowest amount of alcohols (18.4 ± 0.1) and the highest value of aldehydes (29.6 ± 0.3), mostly represented by hexanal (18.5 ± 1.0), were recorded (Table 5).

Similarly, aldehydes (22.6 ± 1.6) were among the most represented chemical classes in Senatore Cappelli VOCs profile along with organic acid (25 ± 2), and alkanes (20.4 ± 2.3). Also in Senatore Cappelli VOCs profile, hexanal (10.0 ± 0.5) was the most abundant aldehyde, while alkanes were mostly represented by tetradecane (11.3 ± 0.8).

In old Saragolla and Ofanto, the most represented chemical classes were organic acids (old Saragolla: 33.9 ± 1.4 ; Ofanto: 32.5 ± 2.7), and alcohols (old Saragolla: 27.7 ± 1.5 ; Ofanto: 27.0 ± 0.5), mostly represented by 2,3-butanediol (9.4 ± 0.5) and benzyl alcohol (6.8 ± 0.2), respectively. Alcohols (26.2 ± 1.8), in particular 1-hexanol, 2-ethyl- (10.1 ± 1.9), and organic acids (19.4 ± 4.1) were the most represented chemical classes also in Mec VOCs profile. Moreover, in this variety the highest value of benzene derivatives, only represented by *p*-Xylene (11.8 ± 1.9), was recorded compared to the other varieties.

4. Discussion

Control of stored-product insect pests for many years was mostly based on the use of synthetic insecticides, in particular phosphine and methyl bromide (Taylor, 1994; Hagstrum and Phillips, 2017). However, the repeated use of chemicals has resulted in the “3 R” problems of Resurgence, Resistance, and Residual effects with negative consequences on human health, environment and no target organism (Parkash et al., 2023). The present study aimed to identify potential source of bioactive compounds and un-attractive genotypes to be used in future breeding programs.

The granary weevil adults showed positive and significant RIs indicating an effective attraction in response to odours of all varieties tested. However, among some of them, significant differences were recorded. For both sexes of *S. granarius*, the Faridur modern variety showed the higher RI. Analysis of variance did not highlight significant differences in males. Whilst, in females the RIs to Faridur, Ofanto, Mec, and old Saragolla were significantly higher than that to Svevo variety. These observations are in accordance with results from a previous study that investigated the antennal sensitivity of *S. granarius* males and females to a wide range of cereal volatiles by electroantennography (Germinara et al., 2002). In that study, the cluster analysis found a greater number of EAG response groups in females than in males. The greater ability of females to discern odours of different food substrates could be associated with a higher need to carefully select sites not only for feeding but also for mating and oviposition (Germinara et al., 2002).

In the case of *R. dominica*, both sexes exhibited a significant preference for Faridur, Mec, and old Saragolla kernels. On the other hand, odours from the modern varieties Ofanto and Svevo and the old varieties Senatore Cappelli and Dauno III did not elicit a significant attraction in *R. dominica* beetles. It is notable that in the no-choice susceptibility tests, Faridur variety, characterized by a lower kernel hardness, showed a greater susceptibility to both *S. granarius* and *R. dominica* compared to all the varieties tested in this study (D'Isita et al., 2024).

Most of the VOCs identified in the present study were already found by other authors (Maga, 1978; Zhou et al., 1999; Mattiolo et al., 2017; Germinara et al., 2019; De Flaviis et al., 2021, 2022, 2023; Bu'sko et al., 2010). Among the seven genotypes, some differences in the VOCs profile regarding the relative abundance of chemical classes and compounds were recorded; however, according to the HCA analysis, it was not possible to discriminate the old and modern varieties studied by volatile profile, contrary to a previous study (De Flaviis et al., 2023). Faridur, the most susceptible and attractive variety for both species, was rich in alcohols, alkanes and terpenes. In old Saragolla, Ofanto and Mec the most represented chemical classes were alcohols and organic acids. Dauno III was the richest variety in alcohols and lactones, and the poorest in aldehydes and terpenes content compared to other varieties. Whilst, Svevo showed the lowest amount of alcohols and the highest value of

aldehydes, Senatore Cappelli VOCs were rich in aldehydes but also in organic acids and alkanes.

In the present study, the olfactory preferences recorded among varieties for both species could be associated with differences in types and concentrations of volatile components and may be affected by synergistic effects of several compounds (Bruce et al., 2005; Najar-Rodriguez et al., 2010; Webster et al., 2010; Bruce and Pickett, 2011; Cha et al., 2011). Different cereal VOCs, particularly some alcohols and short-chain aliphatic aldehydes, are detected by antennal sensilla of *S. granarius* and elicit significant behavioural responses (Maga 1978; Zhou et al., 1999; Germinara et al., 2002, 2008, 2019; Piesik and Wenda-Piesik, 2015; Trematerra et al., 2021; Cao et al., 2023). Among alcohols, 1-hexanol acted as a repellent, instead, 1-pentanol showed a dual activity as attractive at lower concentrations and repellent at higher ones (Germinara et al., 2008). 1-octanol was reported as an egg-laying stimulant for *S. granarius* females (Niewiada et al., 2005). Furthermore, 2-ethyl-hexanol is considered a potential attractant for another important storage insect species, *Callosobruchus maculatus* (F.) (Coleoptera, Curculionidae) (Ajayi et al., 2015). By contrast, aldehydes could disrupt the granary weevil orientation to kernels of attractive commercial wheat varieties (Germinara et al., 2015) and could also inhibit the granary weevil orientation to pigmented wheat genotypes (Germinara et al., 2019). In particular, hexanal and heptanal showed a strong repellent effect against adult *S. granarius* adults (Germinara et al., 2008). As a consequence, the host finding by *S. granarius* could be considered a complex process based on the balance of positive and negative odour stimuli (Germinara et al., 2008). Moreover, Piesik and Wenda-Piesik (2015) showed that *S. granarius* adults were not attracted by a blend of aliphatic aldehydes emitted by cereal grains. In this regard, the higher content of aliphatic aldehydes in the VOCs profile of the Svevo variety could at least partly explain their lower attractiveness towards granary weevil adults.

The grain hardness reflects the degree of adhesion between the starch granules and the protein matrix (Swan et al., 2006). It is notable that Faridur, the most attractive variety to granary weevil, and Svevo, the lowest attractive one, genetically differ only for puroindoline (*Pina* and *Pinb*) genes regulating kernel hardness (Pasqualone et al., 2023). In this study, the two genotypes differed significantly for some classes of compounds. This strongly suggests that the cohesive forces between molecules of the matrix influence the production of VOCs.

During the host finding process, also *R. dominica* uses olfactory cues to discriminate between volatiles from suitable and unsuitable plants, with a strong olfactory preference for wheat substrates which provided the maximum reproductive success (Potter, 1935; Bashir, 2002; Edde and Phillips, 2006; Cao et al., 2024). However, to the best of our knowledge, little is known about the role of chemical classes or specific VOCs on the orientation of Bostrichidae, and in particular of *R. dominica* to host-plant.

In conclusion, this study is the first attempt to evaluate attractiveness of old wheat varieties towards *S. granarius* and *R. dominica*; moreover, it provides new knowledge useful for further investigation testing, in behavioral bioassays, the VOCs of the most and the lowest attractive varieties. In fact, defining the biological activity of these VOCs could offer new insights into the mechanism of host finding by both pests. From a practical perspective, this information would be useful to identify bioactive compounds valuable in the development of effective attractants for semiochemical-based control means to enhance the performance of aggregation pheromone-based lures (Chambers, 1990; Dowdy et al., 1993; Dissanayaka et al., 2020; Morrison III et al., 2023) or strong repellents deterring host finding by pests (Germinara et al., 2008, 2012). Furthermore, clarifying the possible role of VOCs in the mechanisms involved in semiochemical interactions between host-plant and postharvest insect pests, is crucial for new breeding programs aiming to develop new varieties producing high levels of repellents that increase the storability of wheat grains.

CRediT authorship contribution statement

Ilaria D'Isita: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Onofrio Marco Pistillo:** Investigation, Formal analysis. **Federica Lo Muzio:** Investigation, Formal analysis. **Sandra Pati:** Writing – review & editing, Formal analysis. **Antonella Marta Di Palma:** Writing – review & editing. **Pasquale De Vita:** Writing – review & editing, Resources, Investigation, Formal analysis, Conceptualization. **Giacinto Salvatore Germinara:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ilaria D'Isita reports financial support was provided by Italian Ministry of University and Research (Ph.D. scholarship - code number DOT13YISJ8). Giacinto Salvatore Germinara reports financial support was provided by Italian Ministry of University and Research (PRIN project 2022 Prot. 202282ZTPL). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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13. General discussion and conclusion

Insect infestation represents one of the main constraints in postharvest systems, causing not only direct feeding losses but also contamination, quality deterioration, enhanced fungal growth, and reduced market value (Rajendran, 2002; Neethirajan et al., 2007; Hagstrum et al., 2012; Kumar, 2017; Rajendran, 2020; Srivastava and Mishra, 2021). Among the most economically important pest species in temperate and Mediterranean regions, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) and *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) occupy complementary ecological niches, acting respectively as a primary internal feeder of intact kernels and as a secondary pest of processed cereal products (Rees, 2004; Plarre, 2013; Trematerra et al., 2012).

For decades, postharvest pest management has relied predominantly on synthetic fumigants, primarily phosphine (PH₃), and residual contact insecticides belonging to the pyrethroid and organophosphate classes (Fields and White, 2002; Lorini et al., 2007; Daglish et al., 2018). Resistance to phosphine and other conventional insecticides has been documented in major stored-product pests worldwide, reducing the reliability of control measures that for many years were considered the backbone of postharvest protection (Boyer et al., 2012; Nayak et al., 2020; Agrafioti et al., 2024). Regulatory restrictions on active substances and increasing consumer demand for residue-free food products have further reduced the availability of effective chemical tools (Fields and White, 2002; Isman, 2015; Handford et al., 2015; Hamel et al., 2020; Guru et al., 2022).

These developments have accelerated interest in low-impact control tools compatible with Integrated Pest Management (IPM), including botanicals, inert dusts, and semiochemical-based approaches (Golob, 1997; Cox, 2004; Pavela, 2016; Isman, 2020; Ntalli et al., 2021; Prakash et al., 2022; Adimas et al., 2024).

In this scenario, plant by-products have been investigated as sources of bioactive compounds with documented insecticidal activity (Velasques et al., 2017; Campolo et al., 2017; Isman, 2020; Prakash et al., 2022; Bruce and Boateng, 2025; Viswakethu et al., 2025). This PhD thesis mainly focused on the evaluation of processing by-products, specifically pomegranate (*Punica granatum* L.) peels and artichoke (*Cynara cardunculus* var. *scolymus* L.) outer bracts, as potential sources of bioactive compounds for stored-product insect control. The insecticidal potential of pomegranate peel extracts obtained with solvents of different polarity was investigated to identify the most effective extraction conditions and to characterize their effects on insect biology (Chapter 5). The study was then extended to *E.*

kuehniella larvae to verify whether the observed effects were maintained across insect species with different feeding behaviour and ecological roles (Chapter 6).

To identify the compounds responsible for the observed bioactivity, a bioassay-guided fractionation approach was carried out, allowing the isolation of a sterol-like compound and providing insights into its potential mode of action (Chapter 7). In parallel, artichoke outer bracts were evaluated, as little is known about the insecticidal activity of this part of the flower head (Chapter 8).

Given the well-documented abundance of phenolics, tannins and flavonoids in pomegranate peels, and phenols, inulin, fibers, and minerals in artichoke bracts, a strong insecticidal activity could have been initially hypothesised for both matrices (Lattanzio et al., 2009; Pandino et al., 2011; Francavilla et al., 2021; Mo et al., 2022; Carpentieri et al., 2022; Siddiqui et al., 2024). However, the first important conclusion emerging from the core of the thesis is that the biological potential of processing residues cannot be predicted solely on the basis of their phytochemical richness. The results obtained clearly demonstrated that biological efficacy depends on a more complex interplay of factors, including extraction methodology, compound accessibility, mode of exposure, and matrix structural properties. Pomegranate peel emerged as the most promising matrix, particularly when extracted with acetone. Its activity was not restricted to contact toxicity, but extended to feeding deterrence, repellency, and nutritional impairment in both *S. granarius* adults and *E. kuehniella* larvae (Chapters 5 and 6).

The observed reduction in relative consumption rate, relative growth rate, food conversion efficiency, and feeding acceptance indicates that the acetone extract exerts effects on multiple biological functions. This multi-target profile is particularly advantageous in a IPM context, as compounds affecting multiple biological processes may affect insect populations more broadly and may reduce the risk of rapid resistance development than molecules acting through a single target site (Isman, 2015; Al Naggar et al., 2025; Pobożniak and Olczyk, 2025; Komala et al., 2025). Moreover, the consistent activity observed across both species is particularly relevant from an applied perspective, as storage environments frequently host mixed infestations (Athanasios et al., 2011; Mahroof and Hagstrum, 2012; Lampiri et al., 2025). A second major finding concerns the critical importance of extraction methodology. In both *S. granarius* and *E. kuehniella*, acetone extract consistently outperformed *n*-hexane and diethyl ether extracts, indicating that compounds of intermediate polarity are more closely associated with the observed bioactivity (Chapters 5 and 6). This is consistent with previous studies showing that the extraction solvent strongly influences the classes of

compounds recovered and, consequently, the biological outcome (Lee et al., 2024; Chavan et al., 2026).

Further insight into this aspect was provided by the bioassay-guided fractionation approach (Chapter 7). The progressive concentration of biological activity into a single subfraction showed that, although the crude pomegranate peel extract displayed a complex and multifunctional profile, a limited number of compounds may account for a substantial part of the observed effects. The identification of a sterol-like compound as the dominant component of the active subfraction contributes to clarifying the chemical factors associated with the observed bioactivity.

The comparison with commercial β -sitosterol revealed only a partial overlap in activity, indicating that structurally related phytosterols may differ in their biological effects. This suggests that even minor variations in molecular structure, including stereochemistry, can result in markedly different biological outcomes (Tanaka, 2015; Arantes et al., 2024). In this context, sterol-like compounds from the sitosterol family may represent an underexplored source of bioactive molecules with potential applications in stored-product pest management. Although these compounds have been studied mainly from a nutritional and physiological perspective, comparatively little attention has been paid to their potential role as behaviour-modifying or toxic agents in insects (Behmer and Nes, 2003; Holken Lorensi et al., 2019; Mishra et al., 2020; Jing and Behmer, 2020; Zhang, et al., 2024).

A different pattern emerged from the bioassays performed with artichoke outer bract extracts (Chapter 8), which showed a considerably more limited bioactivity profile. Although modest contact toxicity was observed in the acetone extract, ingestion assays did not result in significant mortality or nutritional impairment in *S. granarius*, and antifeedant activity remained minimal. The outer bracts are characterised by a highly lignified and fibrous structure, which likely limits both the extractability and the bioavailability of potentially active compounds (Núñez-Gómez et al., 2023; Carullo et al., 2024). This interpretation is consistent with the broader principle that the insecticidal potential of plant-derived matrices depends not only on their chemical composition, but also on their accessibility and mode of exposure to the target organism (Isman, 2020; Souto et al., 2021).

This concept is further supported by the results obtained with pomegranate peel extracts, where the same matrix produced clearly different outcomes depending on the exposure route in both *S. granarius* and *E. kuehniella*. Contact, ingestion, and repellency assays did not yield comparable levels of activity, indicating that plant-derived preparations do not act uniformly across exposure pathways.

These differences can be explained by the distinct physicochemical and physiological processes involved in each route of exposure, including cuticular penetration, volatility, gut uptake, and metabolic detoxification (Athanassiou and Arthur, 2018; Isman, 2020; Chamani et al., 2025).

For this reason, results obtained under a single exposure route cannot be directly generalised to other application scenarios. Therefore, the development of pomegranate peel extracts as grain protectants requires systematic evaluation under different delivery strategies and exposure conditions relevant to storage systems.

A comparable pattern emerged from the study on λ -cyhalothrin against *Alphitobius diaperinus* (Chapter 9), where insecticidal efficacy was found to vary markedly with temperature, food availability, exposure time, and developmental stage. These results confirm that insecticidal performance is not an intrinsic property of a compound, but depends on the interaction between chemical properties, insect physiology, and environmental conditions (Kavallieratos et al., 2015; Ukoroije and Otayor, 2020; Li et al., 2024).

The studies on inert dusts (Chapters 10) provided further evidence that the efficacy of control tools is strongly context-dependent. Both diatomaceous earth and natural zeolite showed high insecticidal activity against different stored-product species, but their performance varied depending on factors such as commodity type, exposure time, and particle properties, in accordance with previous studies demonstrating that physical characteristics of both the insect and the treated substrate play a crucial role in determining the effectiveness of inert dusts (Subramanyam and Roesli, 2000; Arthur, 2003; Baliota et al., 2023; Athanassiou et al., 2025). Moreover, the biochemical analysis of zeolite exposure (Chapter 11) revealed that its mode of action is not limited to physical desiccation but also involves oxidative stress responses and alterations in detoxification pathways, including the glyoxalase system, suggesting that inert dusts may act through more complex mechanisms than previously assumed and opening new perspectives for their optimisation and application in pest management.

The complementary studies included in Chapter 12 provide an additional ecological perspective, showing that cereal genotype traits influence the behaviour, host preference and susceptibility of *S. granarius* and *Rhyzopertha dominica*. These findings broaden the interpretation of sustainable stored-product pest management by highlighting the role of host-related cues alongside plant-derived compounds and inert dusts.

The present PhD thesis has contributed to the advancement of knowledge in stored-product pest management by clarifying the insecticidal potential of plant-derived materials and the

factors influencing their biological efficacy. In particular, the results identified pomegranate peel extracts as a promising source of bioactive compounds, while showing that their activity is strongly affected by extraction method, route of exposure, and environmental conditions. At the same time, the study demonstrated that not all plant residues have comparable potential, as shown by the more limited activity of artichoke outer bracts. This highlights the importance of considering not only chemical composition, but also structural properties and compound accessibility when evaluating plant-based matrices.

The thesis also provided new insight into insect–plant interactions in stored-product systems, including the role of volatile organic compounds in host selection and the context-dependent nature of insect susceptibility. Together, these findings contribute to a broader understanding of how plant chemistry, insect biology, and exposure conditions interact to determine biological activity.

From an applied perspective, the results support the use of low-impact control tools within current IPM approaches. In this respect, the valorization of food-processing residues such as pomegranate peel and artichoke bracts highlights the possibility of combining pest management efficacy with sustainability, in line with circular economy principles.

The knowledge generated during this PhD provides a solid basis for future studies and for the development of more sustainable strategies for the protection of stored products.

14. References

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15. Annexes: Scientific Curriculum

15.1 Education, Research and Professional experiences

1. 2012: Scientific High School Graduation, Liceo Scientifico "Guglielmo Marconi" di Foggia (Foggia, Italy).
2. 2016: BSc in Agricultural Science and Technology (L-25) – University of Foggia, Department of Agricultural, Food and Environmental Sciences (SAFE). Thesis "Caratterizzazione della resistenza ad orobanche in pisello".
3. 01.05.2017 - 01.07.2017: Training and research period abroad (2 months) at the Department of Agriculture, Policy and Development, University of Reading (United Kingdom). Research activity: "Characterization of the protein fraction in *Triticum durum*".
4. 01.07.2019 - 01.09.2019: Training and research period (2 months) at the Institute of Sciences of Food Production (ISPA), Italian National Research Council (CNR), Bari. Research activity: "Biological and molecular identification of *Fusarium* spp."
5. 01.10.2019 - 01.06.2020: Training and research period abroad (8 months) at the Department of Plants and Crops, Faculty of Bioscience Engineering, University of Ghent (Belgium). Research activity: "Characterization of the biosynthetic pathway of fumonisins produced by *Fusarium proliferatum* in *Allium cepa* L."
6. 2020: MSc in Plant Medicine (magna cum laude) (LM-69) - University of Bari "Aldo Moro", Department of Soil, Plant and Food Sciences. Experimental thesis: "Genetic variability in the fumonisin biosynthesis gene cluster of *Fusarium proliferatum* and pathogenic test on onion (*Allium cepa* L.)".
7. 27.07.2021 - 02.01.2022: Research fellowship (6 months) – University of Foggia, SAFE Department, Laboratory of General and Applied Entomology. Research activity: "Evaluation of monitoring devices for the olive fruit fly for the development of a new Decision Support System (DSS)". Research project: "Gestione olivicola attraverso l'uso di innovazione e controllo".

8. 2022-2025: PhD Course in Management of Innovation in the Agricultural and Food System of the Mediterranean Region (Cycle XXXVII) - University of Foggia, DAFNE Department. Curriculum: Valorization of Mediterranean Regional Productions. Scientific Disciplinary Sector AGR/11 - General and Applied Entomology. PhD research project: "Characterization of the insecticidal properties of extracts from processing by-products of *Punica granatum* L. and *Cynara scolymus* L. against *Sitophilus granarius* (L.) (Coleoptera, Curculionidae) and *Ephestia kuehniella* (Zeller) (Lepidoptera, Pyralidae)".
9. 01.09.2023 - 01.12.2023: Training and research period abroad (3 months) at the Department of Crop Science, University of Athens (Greece). Research activity: "Optimizing λ -cyhalothrin for the effective control of *Alphitobius diaperinus*: Temperature and food effect revealed".
10. 01.02.2025 - 01.02.2026: Research fellowship (1 year) – University of Foggia, DAFNE Department. Research activity: PRIN project 2022 “Biological, chemical and genetic bases of granary weevil and purple wheat interactions (GRANARIUS).

15.2 Memberships and IDs

1. ORCID ID: 0000-0003-0853-9644

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2. SCOPUS ID:

59344108500 <https://www.scopus.com/authid/detail.uri?authorId=59344108500>

3. RG page: <https://www.researchgate.net/profile/Federica-Lo-Muzio>

15.3 Scientific contributions

15.3.1 Published articles indexed in SCOPUS/ISI

1. **Lo Muzio, F.**, Eleftheriadou, N., Boukouvala, M.C., Germinara, G.S., Kavallieratos, N.G., 2024. Optimizing λ -cyhalothrin for the effective control of *Alphitobius diaperinus*: Temperature and food effect revealed. *Journal of Stored Products Research*, 109. <https://doi.org/10.1016/j.jspr.2024.102425>
2. D'Isita, I., Pistillo, O.M., Di Palma, A.M., **Lo Muzio, F.**, De Vita, P., Pati, S., Germinara, G.S., 2024. Behavioural responses of *Sitophilus granarius* (L.) and *Rhyzopertha dominica* (F.) to odours of old and modern wheat genotypes. *Journal of Stored Products Research*, 109. <https://doi.org/10.1016/j.jspr.2024.102433>
3. **Lo Muzio, F.**, Pistillo, O.M., D'Isita, I., Iadarola, G., Di Palma, A., De Cristofaro, A., Rotundo, G., Germinara, G.S., 2025. Insecticidal and Repellent Activity of Different Pomegranate Peel Extracts Against Granary Weevil Adults. *Insects*, 16(12), 1222. <https://doi.org/10.3390/insects16121222>
4. **Lo Muzio F.**, Pistillo O.M., D'Isita I., Iadarola G., Rotundo G, Di Palma A, De Cristofaro A., Kavallieratos N., Boukouvala M.C., Germinara G.S. Biological Activity of Pomegranate Peel for the green management of *Ephestia kuehniella* (Lepidoptera: Pyralidae) larvae. *Crop Protection*, 107729 <https://doi.org/10.1016/j.cropro.2026.107729>
5. Pistillo, O.M., D'Isita, I., **Lo Muzio, F.**, Acquaviva, M., Iadarola, G., Coduti, M., Di Palma, A., Germinara, G.S., 2024. Distribution and flight activity of the asparagus moth, *Parahypopta caestrum*, in southern Italy as monitored by pheromone traps. In *EHC2024: International Symposium on Sustainable Vegetable Production from Seed to Health Booster Sources*, 1416, 325-330. <https://doi.org/10.17660/ActaHortic.2025.1416.42>
6. D'Isita, I., Pistillo, O.M., **Lo Muzio, F. et al.** Susceptibility and attractiveness of ancient wheats to *Sitophilus granarius* and *Rhyzopertha dominica*. *J Pest Sci* 99, 75 (2026). <https://doi.org/10.1007/s10340-026-02037-x>

15.3.2 Published articles not indexed in SCOPUS/ISI

1. D'Isita, I., Pistillo, M., **Lo Muzio, F.**, Pati S., Spadanuda P., Fania F., De Vita P., Germinara, G.S 2026. Uno studio su biodiversità cerealicola e soluzioni sostenibili.

15.3.3 In press, under review and submitted articles

1. Maura N. Laus, Mario Soccio, Ilaria D'Isita, **Lo Muzio**, Giorgia Castagnaro, Onofrio Marco Pistillo, Gianluca Paventi, Giacinto S. Germinara First insights into the insecticidal mechanism of natural zeolite against *Sitophilus granarius*: effects on the antioxidant defence system and glyoxalase pathway Submitted for publication to *Scientific reports*
2. **Lo Muzio, F.**, Pistillo, O. M., D'Isita, I., Iadarola, G., Rotundo, G., Di Palma, A., De Cristofaro, A., & Germinara, G. S. (2026). Bioassay-guided fractionation of the active pomegranate peel extract towards two stored-product insect pests. Submitted to *Scientific Reports*
3. **Lo Muzio, F.**, Pistillo, O. M., D'Isita, I., Iadarola, G., Rotundo, G., Di Palma, A., De Cristofaro, A., & Germinara, G. S. (2026). Evaluation of globe artichoke (*Cynara cardunculus* L.) outer bract extracts against *Sitophilus granarius* (L.). Submitted to *Archives of Phytopathology and Plant Protection*

15.3.4 Conference proceedings

1. Pistillo, O.M., D'Isita, I., **Lo Muzio, F.**, Di Palma, A., Germinara, G.S., 2023. Identificazione del feromone sessuale del cosside dell'asparago, *Parahypopta caestrum* (Lepidoptera, Cossidae): indagine chimica, elettrofisiologica e di attrattività in campo. *Atti XXVII CNIE (Congresso Nazionale Italiano di Entomologia)*, Palermo, 12-16 giugno: 52.
2. **Lo Muzio, F.**, D'Isita, I., Pistillo, O.M., Rotundo, G., Germinara, G.S., Di Palma, A., De Cristofaro, A., 2023. Tossicità da contatto di estratti di buccia di *Punica granatum* (L.) verso adulti di *Sitophilus granarius* (L.). *Atti XXVII CNIE (Congresso Nazionale Italiano di Entomologia)*, Palermo, 12-16 giugno: 172.
3. D'Isita, I., Pistillo, O.M., **Lo Muzio, F.**, Di Palma, A.M., De Vita, P., Germinara, G.S., 2023. Suscettibilità e attrattività di varietà di grano antiche e moderne verso il punteruolo del grano, *Sitophilus granarius* (L.) (Coleoptera, Curculionidae). *Atti XXVII CNIE (Congresso Nazionale Italiano di Entomologia)*, Palermo, 12-16

giugno: 307.

4. Rotundo, G., Paventi, G., D'Isita, I., **Lo Muzio, F.**, Pistillo, O.M., Marconi, E., De Cristofaro, A., Germinara, G.S., 2023. Tossicità per inalazione di farine da semi di *Eriobotrya japonica* Lindl. verso adulti di *Sitophilus granarius* (L.) (Coleoptera, Curculionidae). *Atti XXVII CNIE (Congresso Nazionale Italiano di Entomologia)*, Palermo, 12-16 giugno: 312.
5. **Lo Muzio F.**, Onofrio Marco Pistillo, Ilaria D'Isita, Paolo La Rotonda, Giulia Conversa, Antonella Di Palma, Salvatore Germinara 2024. Insecticidal activity of different extracts from halophyte plants. Meeting dello Spoke 7 “Soluzioni sostenibili per lo sviluppo delle aree marginali”, AGRITECH National Research Center, Bari, Italy, 21–22 November.
6. Pistillo, O.M., D'Isita, I., Lapenda, F., Marino, F., **Lo Muzio, F.**, Iadarola, G., Di Palma, A., Germinara, G.S., 2025. Identificazioni di composti volatili dell'olivo (*Olea europaea* L.) elettrofisiologicamente attivi verso gli adulti di *Philaenus spumarius* (L.). *Atti XXVIII CNIE (Congresso Nazionale Italiano di Entomologia)*, Siena, 16-20 June: 184.
7. Iadarola, G., Pistillo, M.O., D'Isita, I., **Lo Muzio, F.**, Germinara, G.S., Di Palma, A.M., Ladurner, E., Iodice, A., Savino, F., 2025. Valutazione del metodo della confusione sessuale per il controllo di *Prays oleae* (Bernard) in oliveti superintensivi dell'Italia meridionale. *Atti XXVIII CNIE (Congresso Nazionale Italiano di Entomologia)*, Siena, 16-20 June: 187.
8. Pistillo, O.M., Ganassi, S., Lapenda, F., Santagata, I., D'Isita, I., Tedino, C., Di Criscio, D., **Lo Muzio, F.**, Di Palma, A., De Cristofaro, A., Germinara, G.S., 2025. Identificazione di composti elettrofisiologicamente attivi in estratti di adulti di *Philaenus spumarius* (L.). *Atti XXVIII CNIE (Congresso Nazionale Italiano di Entomologia)*, Siena, 16-20 June: 201.
9. Ganassi, S., Pistillo, O.M., Santagata, I., Lapenda, F., D'Isita, I., Di Criscio, D., Tedino, C., **Lo Muzio, F.**, Di Palma, A., Germinara, G.S., De Cristofaro, A., 2025. Identificazione di composti elettrofisiologicamente attivi verso adulti di *Philaenus*

spumarius (L.) in oli essenziali di *Salvia rosmarinus* (Spenn.) e *Lavandula officinalis* Miller. Atti XXVIII CNIE (Congresso Nazionale Italiano di Entomologia), Siena, 16-20 June: 2025.

10. D'Isita, I., **Lo Muzio, F.**, Pistillo, O.M., Pati, S., Di Palma, A., De Vita, P., Germinara, G.S., 2025. Susceptibility and attractiveness of ancient and old wheats to *Sitophilus granarius* (L.) and *Rhyzopertha dominica* (F.). From Seed to Pasta (FSTP) Congress, Bari, Italy, 24-27 September
11. **Lo Muzio, F.**, D'Isita, I., Romanelli, M., Iadarola, G., Laddomada, B., Mancarella, S., Pistillo, O. M., Angione, G., Fania, F., De Vita, P., & Germinara, G. S. (2025). Evaluation of granary weevil utilization efficiency in pigmented wheat varieties. From Seed to Pasta (FSTP) Congress, Bari, Italy, 24-27 September.

15.4 Current editorial tasks

15.4.1 Peer reviewer

1. Archives of Phytopathology and Plant Protection (Taylor & Francis).
2. Journal of Stored Products Research

15.5 Attended congresses, workshops, and meetings

1. Paluzza, 3-7 October 2022

Oral presentation "Bioactivities of plant extracts against some stored-product insect pests". 4th Joint Meeting of Agriculture-oriented PhD Programs at UNIFG, UNICT and UNIUD.

2. Palermo, 12-16 June 2023

Poster "Contact toxicity of *Punica granatum* (L.) fruit peel extracts against the granary weevil, *Sitophilus granarius* (L.) (Coleoptera, Curculionidae)". Co-author of the poster: "Tossicità per inalazione di farine da semi di *Eriobotrya japonica* Lindl. verso adulti di *Sitophilus granarius* (L.) (Coleoptera, Curculionidae)". Co-author of two oral presentations: "Suscettibilità e attrattività di varietà di grano antiche e moderne verso

il punteruolo del grano, *Sitophilus granarius* (L.) (Coleoptera, Curculionidae)" and "Identificazione del feromone sessuale del cosside dell'asparago, *Parahypopta caestrum* (Lepidoptera, Cossidae): indagine chimica, elettrofisiologica e di attrattività in campo". XXVII Congresso Nazionale Italiano di Entomologia (CNIE).

3. Lesina, 30 September - 4 October 2024

Oral presentation "Bioactivity of *Punica granatum* (L.) fruit peel extracts against two stored insect pests". 6th Joint Meeting of Agriculture-oriented PhD Programs at UNIFG, UNICT and UNIUD.

4. Siena, 16-20 June 2025

Oral presentation "Bioattività di estratti di buccia di *Punica granatum* (L.) verso insetti infestanti delle derrate". Co-author of five posters: "Identificazioni di composti volatili dell'olivo (*Olea europaea* L.) elettrofisiologicamente attivi verso gli adulti di *Phlaenus spumarius* (L.)"; "Valutazione del metodo della confusione sessuale per il controllo di *Prays oleae* (Bernard) in oliveti superintensivi dell'Italia meridionale"; "Identificazione di composti elettrofisiologicamente attivi in estratti di adulti di *Phlaenus spumarius* (L.)"; "Identificazione di composti elettrofisiologicamente attivi verso adulti di *Phlaenus spumarius* (L.) in oli essenziali di *Salvia rosmarinus* (Spenn.) e *Lavandula officinalis* Miller". Co-author of one oral presentation: "Suscettibilità e attrattività di grani antichi verso adulti di *Sitophilus granarius* (L.) e *Rhyzopertha dominica* (F.)". XXVIII Congresso Nazionale Italiano di Entomologia (CNIE).

5. Bari, 24-27 September 2025

Poster "Evaluation of granary weevil utilization efficiency in pigmented wheat varieties". Co-author of the poster "Susceptibility and attractiveness of ancient and old wheats to *Sitophilus granarius* (L.) and *Rhyzopertha dominica* (F.)". From Seed to Pasta (FSTP) Congress.

15.5.1 Chairperson activity

Chairperson during the 6th Joint Meeting of Agriculture - oriented PhD Programs. Lesina, September-4 October 2024

Chairperson during the event “Use of new trap devices and the mating disruption method to control the gypsy moth”, organized by the University of Foggia (DAFNE Department) in collaboration with the University of Athens and the University of Thessaly, 20 February 2024.

15.6 Attended courses and seminars

Seminars

1. 4th joint meeting of agriculture-oriented PhD programs UniCT, UniFG and UniUD, Paluzza (UD). 3-7 October 2022
2. “Methods of assessing the quality and safety of packaging - case studies” by Prof. Karolina Wiszumirska, Poznan University (Poland) 11-12-16-18-19 May 2022
3. “OMICS, with a focus on proteomics to understand muscle to meat conversion and discovery of protein biomarkers” by Dr. Mohammed Gagaoua, from Teagasc Food Research Centre, at Ashtown, Ireland. 10 May 2022
4. “Bioactive organic molecules - natural compounds and the methodologies for the synthesis of important heterocyclic compounds” by Dr. Selbi Keskin, Giresun University (Turkey). 25 May 2022
5. “Food contaminants and carcinogenicity and polycyclic aromatic hydrocarbon (PAH): sources, exposure and health effects” by Assoc. Prof. Dr. Sibel Kaçmaz, Giresun University (Turkey) 26 May 2022
6. “Feromoni: le novità sul monitoraggio delle specie aliene e l'evoluzione nella tecnica della distrazione sessuale” by Prof. Giacinto Salvatore Germinara (DAFNE) University of Foggia. 13 March 2023.
7. “Upcoming changes in the packaging industry - proposal on packaging and packaging waste regulation (PPWR)” by Prof. Karolina Wiszumirska, Poznan University (Poland) (Department of Industrial Products and Packaging Quality,

Institute of Quality Science, Poznan University of Economics and Business). 11 May 2023

8. “The possibilities of using intelligent packaging in reducing food waste” by Prof. Mariusz Tichoniuk, (Poland) (Department of Industrial Products and Packaging Quality, Institute of Quality Science, Poznan University of Economics and Business). 12 May 2023
9. XXVII Congresso Nazionale Italiano di Entomologia. PALERMO, 12-16 June 2023.
10. “Trends in analytical techniques for quality and safety controls in the agro-food sector” by Prof. Elson Agastra (University of Tirana). 14 February 2024
11. “Use of new trap devices and the mating disruption method to control the gypsy moth” by Ph.D Maria Boukouvala, Paraskevi Agraftoti, Nikoleta Eleftheriadou, Department of Crop Science (University of Athens). 19-20-21-22 March 2024
12. “Novel Application of gases in food processing and preservation” by prof. Bhesh Bhandari (University of Queensland, Australia) 6 September 2024.
13. 6th JOINT MEETING OF AGRICULTURE-ORIENTED PHD PROGRAMS UNICT, UNIFG AND UNIUD, Lesina (FG). 30 September-4 October 2024.

Courses

1. Statistic course (Part one) by Prof. Fabio Santeramo (DAFNE). June-May 2022
2. Statistic course (Second Part): “Information systems and experimental data processing” by Prof. Marcella Giuliani and Prof. Giuseppe Gatta (DAFNE). May-June 2023
3. Course in “Enzyme assays in plant cell extracts a practical approach” by Prof. Mario Soccio (DAFNE). June-July 2023

4. Course in “Metodologie molecolari utili per il sequenziamento del dna e lo studio dell'espressione genica” by Pro. Concetta Lotti. December 2024- April 2025

International lectures

1. Biology, ecology, and control of *Lymantria dispar* (L.) (Lepidoptera: Erebidiae), a serious pest in forests, urban and suburban areas. PhD Maria Boukouvala, Department of Crop Science, University of Athens, 20 February 2024.
2. The LIFE eGymer project: Using smart traps and pheromones to control the gypsy moth in Europe PhD Paraskevi Agraftoti, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, 20 February 2024.
3. Demonstration of trap devices used for the capture of *Lymantria dispar* adults and larvae, remote monitoring, and mating disruption tools for large-scale management of *L. dispar* PhD Maria Boukouvala, PhD Paraskevi Agraftoti and PhD student Nikoleta Eleftheriadou, University of Athens and University of Thessaly, 20 February 2024.
4. Exploring the *Neoleucopis* spp. complex as prospective biological control agents against *Marchalina hellenica* in invaded forest areas: an overview of their dynamics” PhD student Nikoleta Eleftheriadou, Department of Crop Science, University of Athens, 20 February 2024.

15.7 Member of the organizing team

1. Member of the Organizing Committee of the 6th Joint Meeting of Agriculture-oriented PhD Programs at UNIFG, UNICT and UNIUD, held in Lesina (FG), Italy, from 30 September to 4 October 2024.
2. Organizational support for the informative and experiential event “Insetti a tavola. La rivoluzione è servita! Novel food e le frontiere dell'alimentazione umana e animale”, organized by the Department of Agricultural Sciences, Food, Natural Resources and Engineering in collaboration with the Master in Controllo Ufficiale e Sicurezza Alimentare (CUASA), University of Foggia, 18 March 2025.

15.8 Language certifications and additional courses

1. English language certification - B1 level (Cambridge).
2. Course "Metodologie molecolari utili per il sequenziamento del DNA e lo studio dell'espressione genica" – University of Foggia.

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