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REVIEW

Sweet potato pests: Perspectives on the use of pheromones for control and monitoring

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Abstract

Sweet potato production is mainly concentrated in tropical and subtropical regions, especially in developing countries in Africa, Asia and Latin America. It is a rustic crop fundamental to the population's food security. More research needs to be conducted on the crop, especially on the chemical ecology of insects that affect the plant. This review shows the main pests that affect sweet potato production and presents insect pheromones and their efficiency in semi-field and field tests with different releasers. Small farmers use cultural, biological, and chemical methods to reduce insect pest damage. With the advancement of new research, behavioral control is a growing practice, e.g., sex pheromones are used to monitor and control pests. This practice is possible because of studies on the chemical ecology of insects. There is a need to use specific and safe low-cost strategies associated with IPM. This review aimed to present a compilation of the leading pheromone options for controlling insects that affect sweet potato production, in order to assist in decision-making in the study of new pheromones from known pests and a combination of pheromones with other control strategies.

Keywords: chemical ecology, IPM, pest control, pheromone, semiochemicals

Introduction

The sweet potato, *Ipomoea batatas* (L.) Lam., (Solanales: Convolvulaceae) is an important food crop in tropical regions. Many pest species create significant challenges to its productivity and quality (Kays 2005; Okonya *et al.* 2014). Despite its worldwide importance, sweet potato cultivation still lacks investment in research and development by public institutions, especially in areas such as pest control (Xiao *et al.* 2022).

Chemical ecology, which investigates the chemical interactions between organisms and their environment, has proven to be an effective and practical approach to pest management. It offers alternatives to traditional control methods and should be developed for crop pests.

In this review, the chemical ecology of sweet potato pests was examined, focusing on identifying these individuals and research priorities for pheromone use. The attack of soil and foliage pests affects tuberous roots' productivity and quality. Defoliator insects promote the reduction of leaf area by limiting photosynthesis and causing injuries that facilitate the entry of disease-causing pathogens.

The primary insects that attack the inner part of sweet potatoes are weevils of the Curculionidae family, especially *Cylas formicarius* (Fabr.) (Coleoptera: Brentidae), *Cylas puncticollis* (Boheman) (Coleoptera: Brentidae) and *Euscepes postfasciatus* (Fairmare) (Coleoptera: Curculionidae). In the foliage, the complex of chrysomelids, *Diabrotica speciosa* (Germar) (Coleoptera: Chrysomelidae), *Diabrotica balteata* (LeConte) (Coleoptera: Chrysomelidae), *Diabrotica bivittula* (Kirsch) (Coleoptera: Chrysomelidae),

Sternocolaspis quatuordecimcostata (Lefèvre) (Coleoptera: Chrysomelidae), *Typophorus nigritus* (F.) (Coleoptera: Chrysomelidae), *Paraselenis flava* (L.) (Coleoptera: Chrysomelidae), *Systena* spp. (Coleoptera: Chrysomelidae), *Epitrix* spp. (Coleoptera: Chrysomelidae) and *Chaetocnema apricaria* (Suffrian) (Coleoptera: Chrysomelidae), and caterpillars of the Lepidoptera order and Noctuidae family (*Spodoptera* spp. and *Megastes* spp.) are mainly responsible for the injuries.

Underground pests are considered the most problematic and challenging to control. They cause economic losses even in low numbers because they live underground and continue to attack even during storage (Kapsa 2008). Most of these insects belong to the order Coleoptera, which in the larval stage, cause direct damage to the commercial part of the crop by feeding on the roots, making them unfit for consumption.

Climate change which intensifies the occurrence of crop disease can impact the food security of the world's population (Halsch *et al.* 2021; Skendžić *et al.* 2021; Yasin 2021). Increasing temperatures and changing rainfall patterns can accelerate the life cycle of several species of insect pests, resulting in a more significant number of generations in a shorter period and their rapid dispersion to new areas. Thus, specific and safe integrated pest management (IPM) strategies are needed.

The integrated pest management of sweet potato crops combines cultural, biological, and chemical strategies to minimize damage caused by insect pests. Among cultural practices, crop rotation with grasses interrupts pest reproductive cycles and increases annual yields, while mulching significantly reduces the population of certain species (Mansaray *et al.* 2013; Gurr *et al.* 2016).

Biological control is also a relevant tool in IPM, employing parasitoids and entomopathogenic nematodes against subterranean pests. Nevertheless, chemical control remains widely used, with pyrethroids, neonicotinoids, and organophosphates being the most common active ingredients. In this context, pheromone traps have gained prominence as an ethological strategy within IPM, enabling precise pest population monitoring, mass insect trapping, and mating disruption, thereby reducing reproduction rates.

Using pheromones for IPM in sweet potato culture is a promising strategy for facing the challenges posed by climate change since they reduce the dependence on insecticides and promote more sustainable agricultural practices. The focus in this review of this highly relevant crop was to understand the pests that attack sweet potato crops, by gathering studies on their inter nd intraspecific interactions. At the end of this review, the insect species that affect the sweet potato crop, the available pheromone options, and the perspectives and priorities related to this research topic are discussed.

Importance of sweet potato culture and main limitations to cultivation

Sweet potato (*I. batatas*) is one of the most versatile and economically relevant crops globally, having a strategic role in food security and income generation, especially in developing countries. It can be used for human consumption, animal feed, and as a raw material for food and energy industries. Small farmers represent the majority of producers (Jansson and Raman 2019). In 2020, global sweet potato production was estimated at 92.1 million tons, cultivated in more than 100 countries (FAOSTAT 2021). China leads production, accounting for more than 50% of global supply, at 48.9 million tons. The global sweet potato planting area is estimated to be around 7.2 million hectares. Beyond its direct economic value, sweet potato stands out for its rusticity, high productivity in poor soils, and resistance to adverse weather conditions. It was considered the sixth most important food crop in the world (Alam 2021).

About 821 million people worldwide suffer from malnutrition due to the various impacts of climate change on food security, which necessitates strategies such as low-cost crop production (Wheeler and von Braun 2013). Increasing temperatures can expand pests' geographical distribution, as well as increasing the number of generations per year and the evolutionary process. This in turn hastens the development of insecticide resistance and increases crop damage (Skendžić *et al.* 2021). In this context, sweet potatoes have high nutritional value and resilience to diverse growing conditions, which is essential for food security. However, pest-related challenges which limit yield and quality increasingly threaten their cultivation.

Sweet potatoes have high genetic diversity due to their sexual mode of propagation and the introduction of plants from different origins. Farmers generally select plants with desirable characteristics for reproduction and cultivation using material obtained from previous harvests, seedlings purchased from nurseries or other farmers, and materials from germplasm banks that store and conserve the genetic diversity of plants. These materials have different colors, formats, textures and production yields (Firon *et al.* 2009). Farmers face several production-limiting factors, including a lack of access to financial resources to obtain inputs and production technologies, a lack of information and education on efficient agricultural practices, adverse climate

changes and low availability of quality seed (Okonya *et al.* 2014).

For sweet potato crops, the anticipated appearance of insects and diseases in agricultural environments and the emergence of insects not previously present in the area will require more frequent insecticide applications to reduce production losses. It is estimated that a 2°C increase in temperature can increase insect reproduction from 1 to 5 generations per crop cycle. Warmer periods are associated with an increase in insect abundance in subsequent years, causing significant production losses (Yamamura and Kiritani 1998; Halsch *et al.* 2021).

Global warming affects the dynamics of insect populations, promoting an increase in pests and a decrease in beneficial insects. This scenario leads to the need for alternative strategies for the intensive use of insecticides, such as pheromones. Pheromones can attract and capture pests or interfere with their reproductive behaviors, reducing the need for chemicals. In a warming climate, using pheromones may become even more crucial to sustainably control insect populations and minimize negative impacts on crops and the environment (Deutsch *et al.* 2018).

Use of pheromones in agriculture

IPM aims to control pests efficiently, safely and sustainably. Taking into consideration the characteristics of the environment, culture, and insect population density, it combines different control methods. A modern and widely used method in IPM is the behavioral control of insects using pheromones. This mechanism is considered safe, efficient, and ecologically correct for monitoring and controlling insects. Pheromones have specificity and do not leave residues in the environment. Thus, they are valuable tools for pest control, as they minimize the negative impacts of using traditional synthetic insecticides (Goulart *et al.* 2015). In agriculture, pheromones are used to monitor insects for decision-making when carrying out management. In this phase, a small number of traps are installed per unit area to detect the presence or absence of an insect and monitor its population fluctuation after data collection in the field. The farmer or technician verifies whether the insect is causing economic damage, whether there is population stability and whether natural enemies are present (Goulart *et al.* 2015; Santi *et al.* 2015).

Another way of using pheromones is to control pests, which can be done by mass trap collection to attract the most significant number of individuals to the collection site, consequently reducing the population. An example of success in agriculture is the use of Rhynchophorol®, the aggregation pheromone of the eye borer of the coconut tree *Rhynchophorus palmarum* L. (Coleoptera: Curculionidae), and the primary agent of dissemination of the nematode *Rhadinaphelenchus cocophilus*, the causal agent of red ring disease of the coconut tree (Navarro *et al.* 2002; Goulart *et al.* 2015; Oehlschlager 2016).

Pheromone traps containing Rhynchophorol® can achieve reductions of over 80% in pest populations in treated areas. The effectiveness of control depends on such factors as the pheromone release rate and the type of trap used, with bucket traps being widely adopted for their efficiency and cost-effectiveness. Additionally, capture efficiency is enhanced when traps are combined with sugarcane pieces, coconut palm stems, or pineapple fruits. Moderate release rates (approximately 4.3 mg/day) have been found to be the most economical and effective for capturing *R. palmarum* (Navarro *et al.* 2002).

Sweet potato pests

Considering pest diversity, Jansson and Raman (2019) stated that more than 300 arthropod species can attack sweet potatoes. However, despite this impressive number, only some can cause economic damage.

The pests that attack the sweet potato crop can be divided into two categories: those that attack the foliage and those that attack and feed on tuberous roots. Pests attacking the foliage feed exclusively on leaves, causing significant losses when present in high incidence, especially during the initial stages of the plant. Pests attacking tuberous roots are considered the most notorious because they directly affect the production and commercial value of the crop.

The primary insects attacking the inner part of the sweet potato are weevils of the Curculionidae family, especially *C. formicarius*, *E. postfasciatus* and *C. puncticollis*, which are the most reported in the literature.

In the foliage, the primary pests include the complex of chrysomelids (*D. speciosa*, *D. balteata*, *D. bivittula*, *S. quatuordecimcostata*, *T. nigritus*, *P. flava*, *Systena* spp., *Epitrix* spp. and *C. apricaria*), which competes with several species of defoliating caterpillars of the Lepidoptera order, specifically of the Noctuidae family (*Spodoptera* spp. and *Megastes* spp.), among other secondary pests.

IPM in sweet potato cultivation combines different strategies to minimize damage caused by insect pests. Among cultural practices, crop rotation, mulching, and proper management of crop residues stand out. Studies show that rotations that included grasses, such as bahiagrass *Paspalum notatum* Flügge (Poaceae) and sweet corn *Zea mays* (L.) var. rugosa (Poaceae), result

in higher annual sweet potato yields and significantly reduce pest incidence by interrupting their reproductive cycles, while mulching contributes to reducing the population of *C. formicarius* (Guertal *et al.* 1997; Mansaray *et al.* 2013; Devi *et al.* 2016).

In biological control, the parasitoids *Telenomus remus* and entomopathogenic nematodes of the genera *Heterorhabditis* and *Steinernema* have shown effectiveness against larvae and adults of underground pests, reducing populations by up to 90% under experimental conditions (Ekanayakei *et al*. 2001; Gapasin *et al.* 2016; Fanou *et al.* 2019).

Chemical control is the most common, but its excessive use can lead to pest resistance and negative impacts on non-target organisms. The most commonly used active ingredients include: pyrethroids effective against chrysomelids and other foliage pests, neonicotinoids used for underground pest control, and organophosphates for the control of *D. speciosa*, although their use is being restricted in many countries due to environmental concerns. (Salles and Grutzmacher 1999; Huseth and Groves 2014; Molnar and Rakosy-Tican 2021).

Integrated pest management practices can be employed to regulate insect populations. However, the methods widely adopted in agriculture are relatively new, and the use of insecticides as the primary approach is predominantly highlighted. To promote more sustainable practices from an environmental point of view, it is crucial to adopt ecologically correct procedures, such as behavioral control, which is already used in crops of greater economic relevance.

Pheromones of sweet potato insects

Coleoptera

Weevil larvae are considered a significant problem in culture; however, little is known about their ecology, biology, and forms of control. The larvae penetrate the tuberous roots of the sweet potato, piercing the surface and digging tunnels. This activity causes direct mechanical damage to the roots, weakening their structure and impairing their ability to absorb nutrients and water. Perforations and galleries also allow the entry of pathogens, such as fungi and bacteria, which interfere with root growth and cause yield losses.

Under attack by borers, the sweet potato plant reacts and produces furanoterpenoid ipomeamarone and coumarins Umbelliferone and Scopoletin (Fig. 1) (Uritani *et al.* 1975). Under high infestation, the production of these compounds is increased, causing a characteristic odor and bitter root taste, which reduces its commercial value (Uritani *et al.* 1975; Wang and Kays 2002).

Cylas formicarius

For sweet potato cultures, weevil *C. formicarius* is the most serious pest in the world. Females produce a sex pheromone determined by Heath *et al.* (1986) as (Z)-3-dodecen-1-ol (E)-2-butenoate (Table 1). Glass slides and rubber septa showed similar results in attracting male weevils in the field. Furthermore, higher doses of synthetic pheromone resulted in greater attraction efficiency; in rubber septa, a dose of 1000 ng · 100 μl^{-1} was superior for insect capture.

Yasuda *et al*. (1992) studied the synthetic pheromone of *C. formicarius* in sweet potato fields in Japan. The pheromone effectively attracted male sweet potato weevils for over a month, and the traps were more efficient when placed downwind. Pheromone activity was highest in the late afternoon and early evening and declined rapidly at dawn. Traps set directly on the ground more effectively captured males than those installed above the ground.

Another study evaluated the impact of the sweet potato weevil and the effectiveness of sex pheromone raps in Java, Indonesia. Damage was higher in the dry season, reducing productivity from 3.32 to 2.50 kg · m^{-2}. Pheromone traps showed variations in weekly captures but did not significantly impact losses,

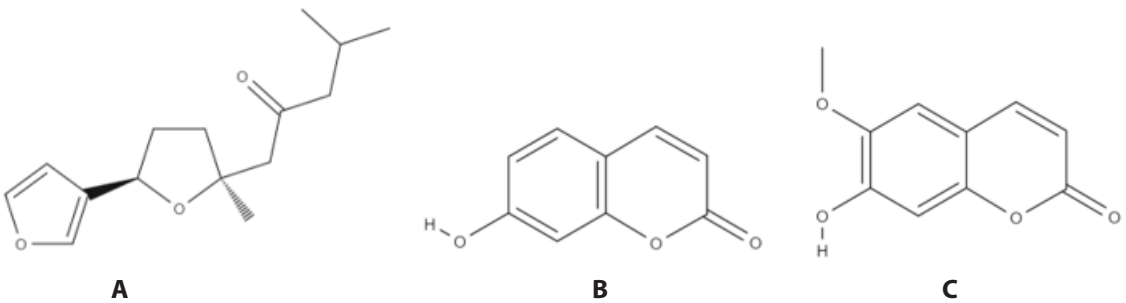


Fig. 1. Compounds with high production in sweet potato tubers after *E. postfasciatus* attack. A – furanoterpenoid ipomeamarone and coumarins: B – umbelliferone and C – scopoletin

with damage of 2.7% and 4.7% caused by the weevil and other pests, respectively. Field trials resulted in an average yield of 2.41 kg · m^{-2}, with captures ranging from 46 to 584 weevils/week, depending on the location (Braun and Van De Fliert 1999).

Cylas puncticollis and C. brunneus

The life cycle and some characteristics of *C. puncticollis* and *C. brunneus* are similar to those of *C. formicarius* (Skoglund and Smit 1994). On average, the incubation period lasts 3–4 days, and the larval instars last 11–14 days. The pupal period ranges from 5 to 7 days, with a total developmental period of 19–24 days. *C. brunneus* has a longer developmental cycle than other species (Musana *et al.* 2016). At 27°C, *C. formicarius* has a total developmental time of about 35–40 days, while *C. brunneus* takes about 44 days to complete its cycle.

Experiments carried out in Uganda and Indonesia led to the identification of the sex pheromones of females of *C. puncticollis* and *C. brunneus* as the compounds decyl (E)-2-butenoate and dodecyl (E)-2-butenoate, respectively (Table 1). Decyl (E)--2-butenoate attracts only *C. puncticollis* males, while dodecyl (E)-2-butenoate attracts males of both species, indicating lower specificity (Table 1) (Downham *et al.* 1999; Vasquez *et al.* 2009).

The effectiveness of pheromone traps for *C. puncticollis* and *C. brunneus* varies depending on the type of dispenser, polyethylene vial or rubber septum. For *C. puncticollis*, polyethylene vials were more effective, while for *C. brunneus*, rubber septa were more selective, capturing fewer *C. puncticollis* individuals. The rate of pheromone release, which is slower in rubber septa, may influence selectivity, but this explanation is not entirely conclusive. Selectivity for *C. brunneus* did not consistently increase with lower initial concentrations and slower pheromone release kinetics, suggesting that factors other than release rate, such as differences in the sensitivity of the two species' olfactory receptors to pheromones, the presence of other unidentified semiochemical compounds, and behavioral and ecological factors, may influence pheromone selectivity (Downham *et al.* 1999).

Pheromone identification represents an advance in the management of these pests, but the lack of specificity of dodecyl (E)-2-butenoate and the influence of the type of dispenser on attracting insects indicate the need for additional research resources to optimize the use of pheromones to control these species.

Euscepes postfasciatus

The sweet potato borer, *E. postfasciatus*, is an essential and vital pest widely reported in South America, Central America, the South Pacific and the Caribbean basins (Shimoji 2011). Its larvae can burrow deep into tubers, similar to *C. formicarius*, promoting high production of the furan-terpenoid ipomeamarone 1-[(2S,5R)-5-(furan-3-yl)-2-methyloxolan-2-yl]--4-methylpentan-2-one and the coumarins umbelliferone 7-hydroxychromen-2-one and scopoletin 7-hydroxy-6-methoxychromen-2-one (Fig. 1). The potato has an unpleasant smell and bitter taste, making it challenging to commercialize (Uritani *et al.* 1975).

Table 1. Pests that attack sweet potato crops and their pheromone compounds

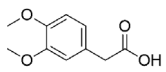
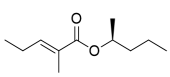
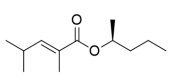
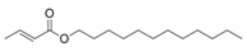
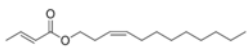
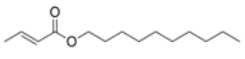
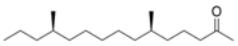
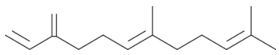
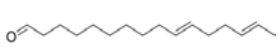
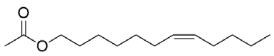
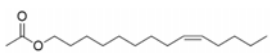
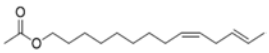
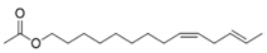
Order/Family/Specie	Pheromones		References
Coleoptera			
Bostrichidae			
Rhyzopertha dominica (Fabricius, 1792)		3:4-dimethoxyphenylacetic acid	Williams *et al.* (1981)
		(S)-(+)-1-methylbutyl (E)--2-methyl-2-pentenoate (S-dominicalure-1)	
		(S)-(+)-1-methylbutyl (E)-2,4-dimethyl-2-pentenoate (S-dominicalure-2)	
Brentidae			
Cylas brunneus (Fabricius)		dodecyl (E)-2-butenoate	Musana *et al.* (2016)
Cylas formicarius (Fabricius, 1798)		(Z)-3-dodecen-1-ol (E)--2-butenoate	Heath *et al.* (1986); Mithran and Subbaraman (1999)

Table 1. Pests that attack sweet potato crops and their pheromone compounds – continuation

Order/Family/Specie	Pheromones		References
Cylas puncticollis (Boheman, 1883)		decyl (E)-2-butenoate	Downham *et al.* (1999); Vasquez *et al.* (2009)
Chrysomelidae			
Diabrotica balteata (LeConte, 1865)		(6R,12R) dimethylpentadecan--2-one	Chuman *et al.* (1987)
Diabrotica bivittula (Kirsch, 1883)		unidentified	Melo *et al.* (2020)
Epitrix sp.	–	unidentified	França and Ritschel (2002)
Sternocolaspis quatuordecimcostata (Lefèvre, 1877)	–	unidentified	Melo *et al.* (2020)
Curculionidae			
Euscepes postfasciatus (Fairmaire, 1849)	–	unidentified	Capinera (2020)
Scarabeidae			
Phyllophaga ephilida (Say, 1825)	–	unidentified	Diagne (2004)
Hemiptera			
Aphididae			
Aphis gossypii (Glover, 1877)		*trans*-β-farnesene	Bowers *et al.* (1972)
Lepidoptera			
Crambidae			
Omphisa anastomosalis (Guenée, 1854)		(10E,14E)-hexadecadienal; (10E,14E-C16Al)	Wakamura *et al.* (2010)
Noctuidae			
Pseudoplusia includens (Walker, 1857)		(7E)-dodecenyl acetate; (7E-12OAc)	Berger and Canerday (1968)
Spodoptera albula (Walker, 1857)	–	unidentified	Broglio *et al.* (2011)
Spodoptera cosmioides (Walk., 1858)		(9Z)-9-tetradecenyl acetate; (9Z)-14:OAc	Blassioli-Moraes *et al.* (2016)
Spodoptera dolichos (Fabricius, 1794)		(9Z,12E)-9,12-tetradecadienyl acetate; (9Z,12E-14:OAc)	Mitchell and Tumlinson (1973)
Spodoptera eridania (Stoll, 1782)		(9Z,12E)-tetradecadien-1-ol acetate; (9Z,12E-14: OAc)	Jacobson *et al.* (1970)
Pyralidae			
Megastes pusialis (Snellen, 1875)	–	unidentified	–
Megastes grandalis (Guenee, 1854)	–	unidentified	–
Sphingidae			
Agrius cingulata (Fabricius, 1775)	–	unidentified	Halder *et al.* (2018)

The tunnels formed can be gateways for other pathogens attacking the crop (Capinera 2020).

The sweet potato weevil demonstrates a weak attraction to LEDs, including chemiluminescent and

ultraviolet lights (Katsuki *et al.* 2012; Nakamoto and Takushi 2001). The effectiveness of capture with LEDs is still insufficient to monitor pests effectively, requiring association with more effective techniques.

E. postfasciatus males exhibit "guarding" behavior both before (pre-copulation) and after (post-copulation) mating. This means that the male remains close to the female, both to guarantee exclusive access to her (before) and to prevent other males from copulating with her (after) (Sato and Kohama 2007; Kumano *et al.* 2009).

Sweet potato weevil males responded to extracts of waxy components obtained from the body surface, exhibiting prolonged mating behavior in glass spheres covered with extracts from females. These responses suggest that body surface lipid components can be used as semiochemicals for pest management (Isa *et al.* 2019). However, the specific compound that generates this response for *E. postfasciatus* has yet to be described.

Phyllophaga ephilida

The white larva *Phyllophaga ephilida* (Say) (Coleoptera: Scarabaeidae) is another crop pest. Adult beetles of the genus are nocturnal and feed on tree leaves, mainly hardwood.

Adult females lay eggs in the soil at 2.5–20 cm depths. After hatching, larvae feed on the roots and stems of plants, including sweet potatoes, and form tunnels that affect the quality and marketing of tubers (Diagne 2004).

Thus far, pheromones against *P. ephilida* have not been identified (Diagne *et al.* 2006).

Chrysomelidae

Members of the chrysomelid beetle complex Coleoptera of the family Chrysomelidae are phytophagous beetles that can cause severe defoliation in the larval and adult stages. Some members of the subfamilies Cassidinae, Eumolpinae and Galerucinae are species that attack sweet potato crops. Chrysomelids have similar behaviors, life cycles and injuries, which makes it challenging to define the impact of each species. Thus, they are divided into a complex composed of the species *D. speciosa, D. balteata, D. bivittula, S. quatuordecimcostata, T. nigritus, P. flava, Systena* spp., *Epitrix* spp. and *C. apricaria* (França and Ritschel 2002; Melo *et al.* 2020).

Of the chrysomelids that affect sweet potato crops, sex pheromones are reported only for *D. balteata*, a minor pest. Therefore, further studies are needed to identify active compounds in the communication of this complex of beetles, aiming at their use in IPM techniques, such as monitoring and control.

Diabrotica balteata

D. balteata is a polyphagous pest. Adults oviposit close to the roots of host plants. After hatching, the larvae feed on the roots, while the adults feed on the leaves, flowers, and fruits. This species does not present diapause in its eggs and has several continuous generations throughout the year, making it a tricky pest to control (Cabrera *et al.* 2020).

Chuman *et al.* (1987) identified the sex pheromone of *D. balteata* as (6R,12R) dimethylpentadecan-2-one (Table 1). Field tests were performed by incorporating the formulated material into filter paper or rubber septum and using it in wing-type traps (Pherocon, California, USA), showing a significant response to the formulation.

Field tests were carried out on sweet potato and pumpkin plantations, with traps containing different concentrations of the synthetic pheromone (1, 4, 10, 100 and 1000 µg on filter paper and 0.03, 0.1, 0.3, 1, 3, 10 and 30 mg in rubber septa). The natural material and a control (hexane) were compared. The optimal dose of synthetic pheromone was 10 µg, which captured an average of 104.2 males per trap, surpassing lower doses (1 µg: 65.5 males) and higher doses (100 µg: 37.2 males; 1000 µg: 8.7 males). In traps with rubber septa, doses between 30 and 300 µg were more effective, with a maximum capture of 24.8 males at 300 µg. It is essential to highlight that research on insect pheromones is complex and requires the combination of different approaches, including chemical, behavioral and ecological studies. The study did not investigate the interaction of the pheromone with other volatile compounds potentially present in the species' chemical communication. Mclaughlin *et al.* (1991) found that the bioactive form of the female sex pheromone of *D. balteata* is (6R,12R) dimethylpentadecan-2-one. The other three stereoisomers ((R,S), (S,R), and (S,S)) did not attract males and did not inhibit the capture of males by the bioactive isomer. Field tests revealed that a 300-µg load of the RR isomer in a rubber septum effectively captured the male. However, capture decreased significantly with bait containing more than 300 µg of the RR isomer.

This result is crucial for developing more efficient pheromone traps for managing *D. balteata*. This indicates that the ideal concentration of the pheromone in the bait is essential to maximize the capture of males. Furthermore, this study highlights the importance of using the correct bioactive form of pheromone, as the other stereoisomers were ineffective. The ideal dose and the influence of stereoisomers still need to be further explored to optimize its practical application.

Lepidoptera

In sweet potato production, lepidopterans are hardly considered more severe than borers because the larvae generally do not reach the commercial part and feed on the crop's leaves. Defoliations cause yield reductions only in severe attacks (Lugojja *et al.* 2001). According to Lugojja *et al*. (2001), it takes more than one defoliation event during the crop cycle for significant yield loss.

Megastes pusialis and M. grandalis

Megastes pusialis (Snellen) (Lepidoptera: Crambidae) and *M. grandalis* (Guenee) (Lepidoptera: Crambidae) are moths from the Crambidae family. The larvae excavate galleries in the collar and stems of plants, especially sweet potatoes, causing significant damage (Cavalcante *et al.* 2013). The females lay eggs at the base of the plant, and the larvae, pink in color with black dots, feed internally, forming galleries. The life cycle lasts around 57 days, and the adults are dark brown moths.

Symptoms of attack include swelling, cracks, and holes in the stems as well as wilting and drying of the branches. The lesions mainly affect the sap-conducting vessels and, occasionally, the roots, resulting in leaf loss, branch wilting and reduced production, which can vary from 30 to 50%. The death of stems is a strong indication of the presence of the pest in the crop (Sorensen 2009).

Omphisa anastomosalis

The caterpillar *Omphisa anastomosalis* (Guenée) (Lepidoptera: Crambidae), which predominantly occurs in Asia, pierces the main stem of the plant and occasionally reaches the storage organ, causing cavities, wilting and eventual plant death (Ohno *et al.* 2010). It is considered the second most important pest of sweet potatoes in areas where it coexists with sweet potato weevils. Infestations by *O. anastomosalis* can cause significant production losses, ranging from 30 to 50%. Controlling the pest is difficult because its immature stages feed inside the plant's branches (McQuate *et al.* 2019). For *O. anastomosalis*, Wakamura *et al*. (2010) identified an active compound obtained from the extract of pheromone glands taken from the abdomens of virgin females (10E,14E)-hexadecadienal (Table 1). In field tests, the synthetic sex pheromone was less attractive than the use of virgin females.

McQuate *et al.* (2019) investigated the efficacy of a combination of sex pheromones (10E,14E)-10,14-hexadecadienal (Type I) and (3Z,6Z,9Z)-3,6,9-tricosatriene (Type II) to monitor and control the *O. anastomosalis* in Hawaii. Field tests demonstrated that adding the Type II component significantly increased the capture of males in traps by up to 13 times compared to using the Type I component alone. The ideal Type I: Type II ratio ranged between 1 : 5 and 1 : 10. Additionally, the 12-week weathering study revealed that bait attractiveness persisted throughout this period, gradually decreasing the Type I: Type II ratio.

Family Noctuidae and the Spodoptera Complex

The Noctuidae family constitutes one of the most diverse groups of moths, and most pests of agricultural importance are included in this family. When small, the caterpillars scrape the leaves, causing superficial damage without loss of production. However, they can evolve into lesions with regular edges. Reaching their last instar, they can destroy the leaves and even the thinnest stems (Rabieh 2018).

In South America, the Spodoptera complex is formed by essential pests for several commercial crops, such as corn, soybean and cotton. The main species of the complex include *Spodoptera albula* (Walker) (Lepidoptera: Noctuidae), *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae), *Spodoptera eridania* (Stoll) (Lepidoptera: Noctuidae), and *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae). *Spodoptera dolichos* (Fabr.) (Lepidoptera: Noctuidae), *S. albula* and *S. cosmioides* have been reported in sweet potato crops. Despite the large number of species present and their importance in other crops, these are considered secondary pests for sweet potato, as they do not attack the commercial part of the sweet potato, hardly causing any economic damage.

Among the reported species of the Noctuidae family, the sex pheromones of have been identified as follows: *S. dolichos*: (9Z,12E)-9,12-tetradecadienyl acetate (Mitchell and Tumlinson 1973), *S. cosmioides*: (9Z)-9-tetradecenyl acetate (Blassioli-Moraes *et al.* 2016), *S. eridania*: (9Z,12E)-tetradecadien-1-ol acetate (Jacobson *et al.* 1970), and *P. includens*: (7E)-dodecenyl acetate (Table 1).

Other defoliating caterpillars of the Noctuidae family have been identified in sweet potatoes in Brazil: *Pseudoplusia includes* (Walk.) (Lepidoptera: Noctuidae) and *Agrius cingulata* (Fabr.) (Lepidoptera: Noctuidae) (Broglio *et al.* 2011).

Hemiptera

Aphis gossypii

Aphis gossypii (Glover) (Hemiptera: Aphididae) is an aphid that damages sweet potato crops, as it transmits phytoviruses and reduces plant vigor by sucking sap and injecting toxins. Although not considered a key pest, its chemical control can be counterproductive,

since it eliminates the natural enemies that regulate its population. Therefore, it is essential to know the biology and ecology of this insect to develop IPM strategies (Ames *et al.* 1997).

The sex pheromone of *A. gossypii* has not yet been described. However, an alarm pheromone has been reported for aphids, triggering the dispersal and evasion of individuals of the same species when attacked by another, such as a natural enemy. They identified the compound as trans-β-farnesene (Table 1) (Bowers *et al.* 1972).

(E)-β-Farnesene triggers dispersal and evasion responses. This characteristic can be exploited in pest control strategies, using the alarm pheromone to manipulate the behavior of aphids, inducing colonies' dispersal and reducing crop infestation. Furthermore, (E)-β-farnesene can also act as a kairomone, recruiting natural enemies of aphids, such as ladybugs and lacewings, aiding in biological control (Bushra and Tariq 2014).

Storage pests

Pests that attack sweet potatoes in storage are a severe problem due to the resulting economic losses. Without monitoring and control measures, the injuries can be even greater than those incurred in the field. Some studies have reported the identification and use of pheromones to monitor and control these species (Hackman *et al.* 1948).

Rhyzopertha dominica

Rhyzopertha dominica (Fabr.) (Coleoptera: Bostrichidae) is a pest that attacks stored grains and has a reddish brown to dark brown color (Edde 2012). It can attack dried sweet potato roots and affect their quality (Mihale *et al.* 2009). *Rhyzopertha dominica* is a holometabolous insect, and its life cycle lasts an average of 60 days, with an egg incubation period of 15 days, a larval period of approximately 22 days, a pupal stage of 5 days and an adult stage of around 29 days (Reddy 2015).

Hackman *et al.* (1948) determined the phenolic substances released through the cuticle in six arthropod species, including *R. dominica*. The observed compound was 3:4-dimethoxyphenylacetic acid (Table 1). Further research has revealed that the main components of the aggregation of pheromone in *R. dominica* are comprised of (S)-(+)-1-methylbutyl (E)-2--methyl-2-pentenoate and (S)-(+)-1-methylbutyl (E)-2,4-dimethyl-2-pentenoate, named dominicalure 1 and 2, respectively (Williams *et al.* 1981).

Sweet potato resistance to pest attack

Weevils are the primary pests attacking the commercial parts of sweet potatoes. Therefore, it is necessary to adopt control measures associated with IPM and to determine the plant's defense mechanisms against attacks by these pathogens. Some studies have investigated the chemical basis of sweet potato resistance to weevil attacks to identify the compounds responsible for this resistance present in different cultivars (Muyinza *et al.* 2012; Anyanga *et al.* 2013, 2017).

Chemical analyses of sweet potato varieties have shown differences in the levels of certain compounds, such as flavonoids and alkaloids, between resistant varieties (New Kawogo and Tanzania). Compounds present in sweet potato latex and on the root surface can cause oxidative stress and impair weevil protein absorption, consequently affecting their development. This study provides important insights into the chemical basis of sweet potato resistance. It has potential applications in developing new strategies to control sweet potato weevil *C. puncticollis* (Stevenson *et al.* 2009).

Field trials and laboratory bioassays comparing seven resistant and three susceptible sweet potato varieties have revealed that resistance is not just a way to avoid damage but an active process. Higher concentrations of caffeic and coumaric acid esters, including hexadecyl, heptadecyl, octadecyl and quinic acid, are correlated with resistance in sweet potato varieties.

Octadecyl coumarate and octadecyl caffeate compounds are esters of hydroxycinnamic acids found on the surface and in the root epidermis of sweet potato varieties resistant to the weevils *C. puncticollis* and *C. brunneus* (Anyanga *et al.* 2013). These compounds act as insect repellents or inhibitors of feeding and oviposition. Surface applications on susceptible sweet potato varieties significantly reduced weevil damage in laboratory tests, suggesting that weevils are involved in plant resistance (Anyanga *et al.* 2013).

Currently, the New Kawogo and Tanzania varieties stand out in research and are considered the most resistant (Stevenson *et al.* 2009; Anyanga *et al.* 2013). Despite several studies on the resistance of sweet potato varieties to weevils, none are effectively immune to insect attacks. The compounds present in resistant varieties help to reduce damage. The presence of high hydroxycinnamic acid ester concentrations in the epidermis and root surface indicates their relationship to resistance to attack and oviposition by adult insects due to its restriction to the root surface; the larvae are not affected, and therefore, the internal area that suffers the most damage is not protected by the presence of these compounds (Anyanga *et al.* 2021).

Hydrocinnamic acid esters are organic compounds found in many plants, including fruits, grains, vegetables, and herbs, and are an essential part of plant defense systems. They are formed by combining cinnamic acids, such as caffeic acid and ferulic acid, with hydroxylated esters, such as methyl esters (Sova and Saso 2020). They can protect plants from environmental

stress and pathogen attacks and have antimicrobial, antifungal and deterrent properties against herbivores.

Phytoalexins are organic compounds produced by plants in response to biotic (such as pathogen attacks) or abiotic (such as environmental stress) stimuli. These compounds are produced at sites of infection or damage and are essential for defense against pathogens and protection against damage caused by abiotic stress. Several biosynthetic routes make phytoalexins, which can be classified into terpenoids, flavonoids and alkaloids. Occasionally, their biosynthesis occurs via multiple pathways (Akagi *et al.* 2014; Kariya *et al.* 2020).

The main compounds identified in the roots and associated with weevil resistance are hexadecyl caffeic acid, hexadecyl coumaric acid, heptadecyl caffeic acid, octadecyl caffeic acid, octadecyl coumaric acid, and the furan-terpenoid phytoalexin ipomeamarone (Uritani *et al.* 1975; Stevenson *et al.* 2009; Anyanga *et al.* 2013). The information presented highlights the importance of organic compounds, such as phytoalexins and hydrocinnamic acid esters, in protecting plants against pathogens (Figure 1).

Conclusions and future perspectives

It is essential to consider farmers' perceptions and practices when developing insect control solutions for root tuber production. This work guides future research aimed at pest recognition and identifying, synthesizing, and using semiochemicals for pest monitoring and control as part of integrated crop management.

Using pheromones in sweet potato pest management has shown promising results, but significant challenges remain. Identifying pheromones for critical species, such as *E. postfasciatus* and *P. ephilida*, is a priority. Furthermore, studies are needed that address the optimization of traps and releasers, the combination of pheromones with other control strategies and the evaluation of the effectiveness of these approaches under different field conditions. The successful implementation of these technologies requires a deeper understanding of pest-specific life cycles and behaviors.

The main pheromone options currently available are highlighted for the management of pests that affect sweet potato crops, such as *C. formicarius*, *C. puncticollis*, *C. brunneus*, *O. anastomosalis*, *S. cosmioides*, *S. eridania*, *S. dolichos*, *P. includens*, *D. balteata,* and *A. gossypii*. The identification and synthesis of pheromones for other vital pests, such as *E. postfasciatus*, represent current research priorities. Furthermore, studies on the optimization of traps, releasers, the combination of pheromones with other control strategies and the evaluation of effectiveness in different field conditions are necessary to obtain more robust results.

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REVIEW

Regulatory differences in crop protection systems for soybean production: implications of the EU–Mercosur trade agreement

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Abstract

The political conclusion of the EU–Mercosur Partnership Agreement in December 2024 marks a significant shift in global agri-food trade and raises fundamental questions on regulatory coherence in plant protection. This study provides a comprehensive comparative assessment of crop protection systems in soybean (*Glycine max* L.) production between the European Union (represented by Poland) and Mercosur countries. Soybean was selected due to its strategic importance in global feed supply chains, the EU's structural import dependence, and the crop's high reliance on chemical and biological plant protection products (PPPs). The analysis reveals pronounced asymmetries in the availability of authorized active substances. Mercosur producers have access to 96 herbicide active substances compared to 16 in Poland (ratio 6 : 1); 96 chemical fungicide active substances compared to 5 (19 : 1); and 94 chemical insecticide active substances compared to only 2 (47 : 1). The disparity is particularly striking for biological fungicides, where 104 microbial strains are registered in Mercosur versus 2 in Poland (52 : 1). Even in biological insecticides, the ratio remains 3 : 1 (9 vs. 3). Approximately 100 active substances used in Mercosur soybean production are not approved in the EU. Pesticide application intensity in Brazil (12.63 kg a.s./ha) is 4.7 times higher than the EU average and over seven times higher than in Poland. In parallel, Maximum Residue Limits (MRLs) for selected substances differ substantially, in extreme cases by up to 200-fold. These quantitative asymmetries translate into divergent pest management capacity, resistance management flexibility, and production resilience. While the EU regulatory framework reflects a precautionary approach with progressive restriction of active substances, Mercosur systems operate with substantially broader chemical and biological portfolios enabling diversified and rotation-based control strategies. The findings demonstrate that regulatory differences – ranging from 6 : 1 to 52 : 1 depending on product category – constitute a structural factor shaping competitiveness, resistance risk, and food safety governance under the evolving EU–Mercosur trade framework.

Keywords: agricultural policy, international trade, MRLs, pesticide regulation, plant protection products

Introduction

The European Union (EU) and the Mercosur bloc reached a political agreement in December 2024 in an Association Agreement that had been under negotiation for over 25 years (European Commission 2024). This historic agreement establishes one of the world's largest free trade areas, covering a combined population of approximately 780 million people and representing nearly 20% of global gross domestic product (GDP). For the agricultural sector, this agreement carries profound implications, as it will

progressively reduce tariffs and trade barriers on agricultural commodities, fundamentally altering competitive dynamics on global food markets.

The agricultural dimensions of this agreement are particularly significant given the contrasting production systems and regulatory philosophies of the two trading blocs (de Oliveira *et al*. 2024). The European Union has pursued an increasingly precautionary approach to pesticide regulation under the Common Agricultural Policy and more recently the Green Deal and Farm to Fork Strategy, resulting in the withdrawal of numerous active substances from the market (Korbas *et al*. 2017; Strażyński *et al.* 2025). Mercosur countries, conversely, have maintained broader pesticide portfolios to address the intensive pest and disease pressures characteristic of tropical and subtropical agricultural systems. Brazil, a major global agricultural player, ranks among the top five agro-food producers and exporters, making it one of the largest consumers of pesticides worldwide. Notably, approximately 30% of the pesticides used in Brazil are banned in the European Union. Paradoxically, some of these banned agrochemicals re-enter northern markets through imported agro-food products (Bombardi 2019; Perobelli 2025).

This study provides a comprehensive comparative analysis of crop protection systems between the EU (represented by Poland) and Mercosur countries, using soybean (*Glycine max* L.) as the primary model crop. Soybean was selected for several compelling reasons: it represents the dominant crop in Mercosur agricultural production, accounting for approximately 40% of Brazil's total crop output; the EU imports over 50% of its soybean and soybean meal requirements from Mercosur, creating a fundamental structural dependency; the contrasting regulatory approaches are most starkly evident in this crop; and the intensive pest pressure in tropical soybean production has driven registration of an exceptionally broad portfolio of plant protection products, enabling meaningful comparison (Bułkowska and Ambroziak 2025; Gohin and Matthews 2025).

Key drivers of regulatory divergence include differences in risk assessment frameworks, with the EU applying a hazard-based approach leading to the withdrawal of substances due to carcinogenicity, endocrine disruption, persistence, and ecotoxicological risks.

Materials and Methods

Scope and study design

The analysis was based on a comparative assessment of plant protection products authorized for soybean (*G. max*) cultivation in Poland and in Mercosur countries (Brazil, Argentina, Bolivia, Paraguay, and Uruguay). Poland was selected as a representative case for the European Union due to the harmonized nature of EU pesticide regulation and the availability of detailed national registration data.

Data sources

Information on authorized plant protection products and active substances in Poland was obtained from the national register maintained by a competent authority, supplemented by unpublished expert analyses conducted at the Institute of Plant Protection – National Research Institute. Data for Mercosur countries were compiled from official national registers, regulatory agency publications, and pesticide sales and use statistics, particularly reports issued by IBAMA (2025) and FAOSTAT (2024) and internet database HOMOLOGA (2025). Active substances were classified into herbicides, fungicides, and insecticides, with further distinction between chemical and biological products. Microbial biological agents were treated at strain level where national regulations recognize individual strains as separate active substances.

Comparative framework

Comparisons focused on: (i) the number of authorized active substances by functional group; (ii) diversity of modes of action relevant to resistance management; and (iii) regulatory status of substances under EU legislation. Maximum Residue Limits (MRLs) were compared using EU Regulation (EC) No 396/2005 and corresponding Brazilian standards.

Results

The Mercosur Bloc: agricultural characteristics

Overview and agricultural potential

Mercosur (Mercado Común del Sur) comprises Brazil, Argentina, Paraguay, Uruguay, and Bolivia, representing one of the world's most important agricultural production regions. The bloc encompasses approximately 389 million hectares of agricultural land – 2.4 times the agricultural area of the entire European Union. This vast territory, combined with favorable climatic conditions and abundant natural resources, positions Mercosur as a global agricultural powerhouse with production potential that significantly exceeds current output levels.

The agricultural structure of Mercosur differs fundamentally from the European model. While the EU maintains approximately 10.3 million farms with an

average size of 17.4 hectares (the average farm size in Poland is approximately 11 ha), Mercosur agriculture is characterized by large-scale commercial operations, particularly in the dominant soybean and cattle sectors. Brazilian farms in the Cerrado and Matopiba regions frequently exceed 1,000 hectares, enabling economies of scale in mechanization and input application that are rarely achievable in European contexts.

Crop production and GMO technology adoption

Mercosur countries have embraced genetically modified crop technology to an extent unmatched elsewhere in the world. The bloc accounts for approximately 46.5% of global Genetically Modified Organisms (GMOs) crop cultivation area, with Brazil alone representing 26.4% of the world total. GMO soybean varieties, primarily those with herbicide tolerance (HT) and insect resistance (IR) traits, dominate production in all major Mercosur producing countries. In Brazil, over 97% of the soybean area is planted with GM varieties, compared to effectively zero in the European Union where GMO crop cultivation remains restricted to a single approved maize variety grown on limited area in Spain and Portugal.

Soybean production represents the cornerstone of Mercosur agriculture. Brazil is the world's largest soybean producer with an annual output exceeding 150 million tonnes, followed by Argentina with approximately 50 million tonnes. Paraguay ranks as the world's fourth-largest soybean exporter despite its relatively small territory. Collectively, Mercosur accounts for over 50% of global soybean exports, making the bloc the dominant supplier to import-dependent regions including the European Union, China, and Southeast Asia.

Plant protection products market: Mercosur vs. European Union

Market size and sales volume

Brazil has emerged as the world's largest consumer of plant protection products, with annual sales reaching approximately 15–16 billion USD. This represents roughly 20% of the global pesticide market concentrated in a single country. The scale of Brazilian pesticide consumption reflects the combination of vast cultivated area, intensive production systems, year-round pest pressure in tropical conditions, and the dominance of crops requiring substantial chemical inputs.

According to IBAMA (Brazilian Institute of Environment and Renewable Natural Resources) data for 2022, total pesticide sales in Brazil reached 809,722 tonnes of active substance. This extraordinary volume is distributed across herbicides (approximately 60% of total use), insecticides (15%), and fungicides (12%), with the remainder comprising other product categories. The 10 most-sold active substances in Brazil account for over 60% of total consumption, indicating significant concentration around key molecules.

Application intensity comparison

Pesticide application intensity – measured as kilograms of active substance applied per hectare of agricultural land – reveals fundamental differences between production systems (Tab. 1). Brazil's application intensity of 12.63 kg active substance per hectare is 4.68 times the EU average and 7.3 times higher than Poland's intensity. This reflects both the broader portfolio of available products and the more intensive pest and disease pressure in tropical production systems. Even Argentina and Uruguay, with more temperate climates, maintain intensities 2.3–2.7 times the EU average.

Table 1. Pesticide application intensity comparison: Mercosur countries vs. European Union (FAOSTAT 2024)

Country/Region	Application intensity [kg a.s. · ha^{-1}]	Ratio to EU average
Brazil	12.63	4.68×
Argentina	6.33	2.34×
Uruguay	7.35	2.72×
Paraguay	3.98	1.47×
Mercosur average	9.41	3.49×
EU average	2.70	1.00×
Poland	1.73	0.64×

Most-used active substances in Brazil

Analysis of IBAMA sales data reveals the dominant role of a relatively small number of active substances in Brazilian agriculture, with significant implications

Table 2. Ten most-sold active substances in Brazil (2022) and their EU regulatory status (IBAMA 2025)

No	Active substance	Sales 2022 [tonnes]	EU regulatory status
1	Glyphosate	382.784	approved (under review)
2	2,4-D	101.887	approved
3	Mancozeb	50.532	banned (2021)
4	Atrazine	77.029	banned (2007)
5	Paraquat	24.894	banned (2007)
6	Acephate	28.746	banned (2003)
7	Imidacloprid	17.295	restricted (2018)
8	Chlorothalonil	15.127	banned (2020)
9	Mineral oil	43.857	approved
10	Sulfur	38.940	approved

for EU import controls (Tab. 2). Critically, eight of the 10 most-used active substances in Brazilian agriculture are either banned or heavily restricted in the European Union. Glyphosate, representing 47% of total sales by volume, remains under contentious regulatory review in the EU. Atrazine (banned 2004), mancozeb (banned 2021), paraquat (banned 2007), acephate (banned 2003), and chlorothalonil (banned 2019) continue to be applied at scale in Brazilian production systems, with residues potentially present on exported commodities.

Active substances in soybean protection: detailed analysis

Herbicides

Weed control is a critical determinant of soybean yield stability. In Poland, soybean protection relies on 16 herbicide active substances, primarily ACCase inhibitors and a limited number of pre-emergence herbicides, supplemented by glyphosate. In contrast, Mercosur countries have access to 96 herbicide active substances representing a wide range of chemical classes and modes of action. The restricted herbicide portfolio in Poland limits opportunities for rotation of modes of action and increases the risk of resistance development, a phenomenon widely documented for ALS- and ACCase-inhibiting herbicides in European arable systems (Heap 2025; Stankiewicz-Kosyl *et al*. 2020, 2021, 2023). In Mercosur systems, broader portfolios allow for diversified programs combining pre- and post-emergence herbicides, which is particularly important in intensive soybean monocultures.

Weed management represents the most critical aspect of soybean crop protection, as early-season competition can reduce yields by 20–50%. The analysis reveals that Poland has 117 registered commercial herbicide products based on 16 active substances, while Mercosur countries collectively access 96 different active substances. Only seven substances are common to both systems: bentazone, clomazone, clethodim, glyphosate, imazamox, pendimethalin, and propaquizafop.

Typical weed flora also differs between regions and reflects both climatic conditions and management systems. In Poland and under other EU conditions, dominant weed species include *Chenopodium album* and *Amaranthus spp.* In contrast, soybean production systems in Mercosur are increasingly challenged by herbicide-resistant populations, particularly glyphosate-resistant *Amaranthus spp.* and *Conyza spp.* (Procópio *et al*. 2024), which require more diverse and intensive herbicide programs. Despite the limited herbicide portfolio, acceptable weed control may still be achieved under moderate pressure through integrated approaches combining mechanical control and crop rotation.

Herbicide active substances registered in Poland

The Polish herbicide portfolio is dominated byACCase inhibitors (graminicides) (Tab. 3). The availability of broadleaf weed herbicides is notably limited, with bentazone providing the primary option. This narrow spectrum constrains integrated weed management strategies and increases resistance development risk (HRAC 2026).

Table 3. Herbicide active substances registered for soybean protection in Poland (2024) (Source: author's own elaboration based on HOMOLOGA 2025)

Active substance	Chemical group (Mode of Action/HRAC)	Products
Bentazon	Benzothiadiazinones (PSII inhibitors/6)	8
Quizalofop-P-ethyl	FOPs (ACCase inhibitors/1)	6
Cycloxydim	DIMs (ACCase inhibitors/1)	4
Dimethenamid-P	Chloroacetamides (VLCFA inhibitors/15)	3
Fluazifop-P-butyl	FOPs (ACCase inhibitors/1)	5
Clomazone	Isoxazolidinones (DXPS inhibitors/13)	4
Imazamox	Imidazolinones (ALS inhibitors/2)	3
Clethodim	DIMs (ACCase inhibitors/1)	7
Metobromuron	Ureas (PSII inhibitors/5)	2
Pendimethalin	Dinitroanilines (microtubule inhibitors/3)	6
Pethoxamid	Chloroacetamides (VLCFA inhibitors/15)	2
Propaquizafop	FOPs (ACCase inhibitors/1)	4
Prosulfocarb	Thiocarbamates (VLCFA inhibitors/15)	3
Pyraflufen-ethyl	Phenylpyrazoles (PPO inhibitors/14)	2
Thifensulfuron-methyl	Sulfonylureas (ALS inhibitors/2)	3

Comparative analysis: Poland vs. Mercosur herbicides

Mercosur countries have access to numerous substances banned or never registered in the EU, including atrazine, paraquat, and various 2,4-D formulations for herbicide-tolerant varieties. PPO inhibitors (fomesafen, flumioxazin, sulfentrazone, lactofen, saflufenacil) provide powerful broadleaf control options unavailable in Poland (Tab. 4).

Fungicides

Chemical fungicide protection of soybean in Poland is based on five active substances representing four modes

Table 4. Comparative availability of herbicide active substances for soybean: Poland vs. Mercosur (Source: author's own elaboration based on HOMOLOGA 2025)

Active Substance	Chemical group (Mode of Action/HRAC)	BR	AR	BO	UY	PY	PL
2,4-D	Phenoxycarboxylates (auxin mimics/4)	✓	–	–	–	✓	–
Acetochlor	Chloroacetamides (VLCFA inhibitors/15)	✓	✓	–	✓	–	–
Atrazine	Triazines (PSII inhibitors/6)	✓	✓	–	–	–	–
Bentazon	Benzothiadiazinones (PSII inhibitors/6)	✓	✓	✓	–	–	✓
Chlorimuron-ethyl	Sulfonylureas (ALS inhibitors/2)	✓	✓	–	–	–	–
Clethodim	DIMs (ACCase inhibitors/1)	✓	✓	✓	✓	✓	✓
Clomazone	Isoxazolidinones (DXPS inhibitors/13)	✓	✓	–	–	–	✓
Cycloxydim	DIMs (ACCase inhibitors/1)	–	–	–	–	–	✓
Dicamba	Benzoates (auxin mimics/4)	✓	–	–	–	–	–
Diclosulam	Triazolopyrimidines (ALS inhibitors/2)	✓	✓	✓	✓	–	–
Diuron	Ureas (PSII inhibitors/5)	✓	–	–	–	–	–
Fluazifop-P-butyl	FOPs (ACCase inhibitors/1)	✓	–	–	✓	–	✓
Flumioxazin	N-phenylimides (PPO inhibitors/14)	✓	✓	✓	✓	–	–
Fomesafen	Diphenyl ethers (PPO inhibitors/14)	✓	✓	✓	✓	✓	–
Glufosinate-ammonium	Phosphinic acids (GS inhibitors/10)	✓	✓	–	✓	–	–
Glyphosate	Organophosphonate and glycine derivative (EPSPS inhibitors/9)	✓	✓	–	✓	✓	–
Haloxyfop-P-methyl	FOPs (ACCase inhibitors/1)	✓	–	✓	✓	–	–
Imazamox	Imidazolinones (ALS inhibitors/2)	✓	✓	–	–	–	✓
Imazethapyr	Imidazolinones (ALS inhibitors/2)	✓	✓	✓	✓	✓	–
Lactofen	Diphenyl ethers (PPO inhibitors/14)	✓	✓	–	–	–	–
Metobromuron	Ureas (PSII inhibitors/5)	–	–	–	–	–	✓
Metribuzin	Triazinones (PSII inhibitors/5)	✓	✓	✓	✓	–	✓
Paraquat	Pyridiniums (PS I electron diversion/22)	–	✓	–	✓	✓	–
Pendimethalin	Dinitroanilines (microtubule inhibitors/3)	–	✓	✓	–	–	✓
Pethoxamid	Chloroacetamides (VLCFA inhibitors/15)	–	–	–	–	–	✓
Propaquizafop	FOPs (ACCase inhibitors/1)	✓	✓	–	✓	–	✓
Prosulfocarb	Thiocarbamates (VLCFA inhibitors/15)	–	–	–	–	–	✓
Pyraflufen-ethyl	Phenylpyrazoles (PPO inhibitors/14)	✓	–	–	–	–	✓
Quizalofop-P-ethyl	FOPs (ACCase inhibitors/1)	✓	✓	✓	–	–	✓
S-metolachlor	Chloroacetamides (VLCFA inhibitors/15)	✓	✓	✓	✓	–	–
Saflufenacil	N-phenylimides (PPO inhibitors/14)	✓	✓	–	–	–	–
Sulfentrazone	N-phenyltriazolinones (PPO inhibitors/14)	✓	✓	✓	✓	–	–
Thifensulfuron-methyl	Sulfonylureas (ALS inhibitors/2)	–	–	–	–	–	✓
Trifluralin	Dinitroanilines (microtubule inhibitors/3)	✓	✓	–	✓	–	–

BR – Brazil, AR – Argentina, BO – Bolivia, UY – Uruguay, PY – Paraguay, PL – Poland

of action, with strong reliance on triazoles and strobilurins. Such a narrow spectrum constrains resistance management and limits response options under high disease pressure, particularly in relation to quinone outside inhibitors (QoI) and demethylation inhibitors (DMI) fungicide resistance development (Lucas *et al.* 2014; FRAC 2025). Mercosur countries have access to 96 chemical fungicide active substances, including multi-site inhibitors, numerous triazoles, succinate dehydrogenase inhibitor fungicides (SDHIs), and combination products (Bombardi 2019; IBAMA 2025). This diversity is essential for managing Asian soybean rust and illustrates how regulatory frameworks influence disease management strategies.

Fungal disease pressure and composition differ markedly between regions. Under Poland and broader

EU conditions, the most relevant soybean diseases include *Sclerotinia sclerotiorum* and *Peronospora manshurica*, which are typically associated with temperate climates and moderate disease pressure. In contrast, soybean production in Mercosur is strongly affected by highly aggressive pathogens such as Asian soybean rust (*Phakopsora pachyrhizi*) and *Cercospora spp.*, which require intensive and often multi-site fungicide programs for effective control (Yorinori *et al.* 2005; Hartman *et al*. 2015).

Fungal disease management in soybean presents the most dramatic asymmetry between regions. Poland has 25 chemical fungicide products based on only five active substances, plus four biological products based on two microbial strains. Mercosur countries collectively access 96 chemical fungicide active substances and 104 biological fungicide strains – ratios of 19 : 1 and 52 : 1, respectively.

Chemical fungicide active substances registered in Poland

Polish fungicide protection relies on QoI strobilurins (azoxystrobin) and DMI triazoles (prothioconazole, difenoconazole) (Tab. 5). These 31 commercial products represent only two primary modes of action, creating significant resistance management concerns.

Table 5. Chemical fungicide active substances registered for soybean protection in Poland (2024) (Source: author's own elaboration based on HOMOLOGA 2025)

Active substance	Chemical group (Mode of Action/FRAC)	Products
Azoxystrobin	Strobilurins (QoI/11)	12
Difenoconazole	Triazoles (DMI/3)	5
Fludioxonil	Phenylpyrroles (M-O-G-D/12)	2
Fluopyram	Benzamides (SDHI/7)	1
Prothioconazole	Triazoles (DMI/3)	11

Comparative analysis: Poland vs. Mercosur chemical fungicides

Mercosur farmers have access to numerous fungicide classes unavailable in Poland, including dithiocarbamates (mancozeb, thiram – withdrawn from EU), benzimidazoles (carbendazim – banned), and multiple SDHI compounds (Tab. 6). Brazil's comprehensive portfolio addresses Asian soybean rust (*Phakopsora pachyrhizi*), requiring preventive multi-site programs impossible with Poland's limited substances.

Biological fungicides

The disparity in biological fungicide availability is particularly pronounced. Poland has only two authorized

Table 6. Comparative availability of chemical fungicide active substances for soybean: Poland vs. Mercosur (Source: author's own elaboration based on HOMOLOGA 2025)

Active substance	Chemical group (Mode of Action/FRAC)	BR	AR	BO	UY	PY	PL
Azoxystrobin	Strobilurins (QoI/11)	✓	✓	✓	✓	✓	✓
Benthiavalicarb	Carbamates (CAA/40)	✓	–	–	–	–	–
Boscalid	Pyrazole carboxamides (SDHI/7)	✓	✓	–	–	–	–
Carbendazim	Benzimidazoles (B-MT-G-ATP/1)	✓	✓	✓	✓	–	–
Chlorothalonil	Chloronitriles (S-E-G-D/M05)	✓	✓	–	✓	–	–
Cyproconazole	Triazoles (DMI/3)	✓	✓	✓	✓	–	–
Difenoconazole	Triazoles (DMI/3)	✓	✓	✓	✓	✓	✓
Dimoxystrobin	Strobilurins (QoI/11)	✓	–	–	–	–	–
Epoxiconazole	Triazoles (DMI/3)	✓	✓	–	✓	–	–
Fludioxonil	Phenylpyrroles (M-O-G-D/12)	✓	✓	✓	✓	–	✓
Fluopyram	Benzamides (SDHI/7)	✓	–	–	–	–	✓
Flutriafol	Triazoles (DMI/3)	✓	✓	–	–	–	–
Fluxapyroxad	Pyrazole carboxamides (SDHI/7)	✓	✓	–	✓	–	–
Isopyrazam	Pyrazole carboxamides (SDHI/7)	✓	–	–	–	–	–
Kresoxim-methyl	Strobilurins (QoI/11)	✓	✓	–	–	–	–
Mancozeb	Dithiocarbamates (S-M-R-D/M03)	✓	✓	✓	✓	✓	–
Metconazole	Triazoles (DMI/3)	✓	✓	–	–	–	–
Myclobutanil	Triazoles (DMI/3)	✓	✓	–	–	–	–
Picoxystrobin	Strobilurins (QoI/11)	✓	✓	✓	✓	–	–
Propiconazole	Triazoles (DMI/3)	✓	✓	✓	✓	–	–

Table 6. Comparative availability of chemical fungicide active substances for soybean: Poland vs. Mercosur (Source: author's own elaboration based on HOMOLOGA 2025) – continuation

Active substance	Chemical group (Mode of Action/FRAC)	BR	AR	BO	UY	PY	PL
Prothioconazole	Triazoles (DMI/3)	✓	✓	✓	✓	✓	✓
Pydiflumetofen	N-methoxyphenyl pyrazolecarboxamides (SDHI/7)	✓	✓	–	–	–	–
Pyraclostrobin	Strobilurins (QoI/11)	✓	✓	✓	✓	✓	–
Tebuconazole	Triazoles (DMI/3)	✓	✓	✓	✓	✓	–
Thiophanate-methyl	Thiophanates (B-M-M-D/1)	✓	✓	✓	✓	–	–
Thiram	Dithiocarbamates (S-E-R-D/M3)	✓	✓	✓	✓	–	–
Trifloxystrobin	Strobilurins (QoI/11)	✓	✓	✓	✓	–	–

BR – Brazil, AR – Argentina, BO – Bolivia, UY – Uruguay, PY – Paraguay, PL – Poland

microbial strains for soybean, whereas Mercosur countries register 104 strains. This diversity enables strain-specific selection and integration of biological products into disease management programs, whereas limited availability in the EU constrains practical implementation of biological control.

The disparity in biological control agents represents the most striking finding. Poland's biological options are limited to *Bacillus amyloliquefaciens* (strains QST713, FZB24) and *Trichoderma asperellum* T34. Brazil registers over 60 *Bacillus* strains and 30+ *Trichoderma* strains, each as separate active substances, providing extensive strain-specific selection for local conditions (Tab. 7).

Table 7. Biological fungicide strains registered for soybean protection: Mercosur vs. Poland (Source: author's own elaboration based on HOMOLOGA 2025)

Microbial species/group	No. of strains	Countries
Bacillus amyloliquefaciens (total)	18	BR, BO, UY, PL
Bacillus subtilis (total)	16	BR, BO
Bacillus velezensis (total)	19	BR, UY
Bacillus pumilus (total)	5	BR
Bacillus licheniformis (total)	4	BR
Trichoderma harzianum (total)	14	BR, BO
Trichoderma asperellum (total)	9	BR, BO, UY, PL
Trichoderma atroviride	2	BR, AR
Trichoderma gamsii	2	BO, UY
Trichoderma koningii	1	BO
Other *Trichoderma* spp.	5	BR, BO, UY
Saccharomyces cerevisiae	1	BR
Plant extracts	4	BR, BO
TOTAL STRAINS	104 (Mercosur) vs. 2 (Poland)	

BR – Brazil, AR – Argentina, BO – Bolivia, UY – Uruguay, PY – Paraguay, PL – Poland

Insecticides

Only two chemical insecticide active substances are authorized for soybean in Poland, providing a very narrow range of modes of action and limiting implementation of insecticide resistance management strategies (Sparks and Nauen 2015; IRAC 2026). Mercosur countries, by contrast, have access to 94 chemical insecticide active substances, supporting diversified and resistance-aware insect pest management strategies.

The spectrum and intensity of insect pest pressure differ substantially between regions. Under Poland and broader EU conditions, the most relevant soybean pests include aphids (*Aphis spp.*), cutworms, and *Sitona spp.*, which are typically associated with moderate and seasonal pest pressure. In contrast, soybean production in Mercosur is affected by more aggressive and economically significant pests such as *Helicoverpa armigera* and stink bugs (*Euschistus spp.*), often requiring intensive and repeated insecticide applications (Bueno *et al*. 2013; Hartman *et al*. 2015; Lamichhane 2017; Oliveira *et al*. 2017).

Insect pest management reveals the most extreme asymmetry. Poland has only 10 commercial insecticide products based on two active substances (cypermethrin, acetamiprid) for soybean. Mercosur countries have access to 94 chemical insecticide active substances – a ratio of 47 : 1 (Tab. 8). Additionally, Poland has four biological products based on three substances, while Mercosur has nine biological insecticide substances (Tab. 9). The Mercosur insecticide portfolio includes chemical classes unavailable in Poland: diamides (chlorantraniliprole, flubendiamide, cyantraniliprole); multiple pyrethroids providing formulation flexibility; spinosyns (spinetoram, spinosad) with reduced environmental impact; organophosphates and carbamates largely withdrawn from EU; and benzoylureas and other insect growth regulators (IGRs) for caterpillar management. Mercosur farmers can implement sophisticated resistance management

Table 8. Comparative availability of insecticide active substances for soybean: Poland vs. Mercosur (Source: author's own elaboration based on HOMOLOGA 2025)

Active substance	Chemical group (Mode of Action/IRAC)	BR	AR	BO	UY	PY	PL
Abamectin	Avermectins (GluCl allosteric modulators/6)	✓	✓	✓	✓	✓	–
Acephate	Organophosphates (AChE inhibitors/1B)	✓	–	–	✓	✓	–
Acetamiprid	Neonicotinoids (nAChR competitive modulators/4A)	✓	✓	✓	✓	–	✓
Alpha-cypermethrin	Pyrethroids (Sodium channel modulators/3A)	✓	✓	✓	–	✓	–
Beta-cyfluthrin	Pyrethroids (Sodium channel modulators/3A)	✓	✓	✓	–	–	–
Bifenthrin	Pyrethroids (Sodium channel modulators/3A)	✓	✓	✓	✓	✓	–
Chlorantraniliprole	Diamides (ryanodine receptor modulators/28)	✓	✓	✓	✓	–	–
Chlorfenapyr	Pyrroles (proton gradient disruptors/13)	✓	✓	✓	–	–	–
Chlorpyrifos	Organophosphates (AChE inhibitors/1B)	✓	–	✓	–	–	–
Cyantraniliprole	Diamides (ryanodine receptor modulators/28)	✓	–	✓	✓	–	–
Cypermethrin	Pyrethroids (Sodium channel modulators/3A)	✓	–	✓	✓	–	✓
Deltamethrin	Pyrethroids (Sodium channel modulators/3A)	✓	–	–	✓	–	–
Diflubenzuron	Benzoylureas (inhibitors of chitin biosynthesis affecting CHS1/15)	✓	✓	✓	–	✓	–
Dinotefuran	Neonicotinoids (nAChR competitive modulators/4A)	✓	✓	✓	✓	–	–
Emamectin benzoate	Avermectins (GluCl allosteric modulators/6)	✓	✓	✓	✓	✓	–
Esfenvalerate	Pyrethroids (Sodium channel modulators/3A)	✓	✓	✓	–	–	–
Fipronil	Phenylpyrazoles (GABA-gated chloride channel blockers/2B)	✓	✓	✓	–	✓	–
Flubendiamide	Diamides (ryanodine receptor modulators/28)	✓	✓	✓	–	–	–
Imidacloprid	Neonicotinoids (nAChR competitive modulators/4A)	✓	✓	✓	✓	✓	–
Lambda-cyhalothrin	Pyrethroids (Sodium channel modulators/3A)	✓	✓	✓	✓	✓	–
Lufenuron	Benzoylureas (inhibitors of chitin biosynthesis affecting CHS1/15)	✓	✓	–	✓	✓	–
Methomyl	Carbamates (AChE inhibitors/1A)	✓	✓	✓	–	–	–
Methoxyfenozide	Diacylhydrazines (ecdysone receptor agonists/18)	✓	✓	✓	✓	–	–
Novaluron	Benzoylureas (inhibitors of chitin biosynthesis affecting CHS1/15)	✓	✓	✓	–	–	–
Profenofos	Organophosphates (AChE inhibitors/1B)	✓	✓	–	✓	✓	–
Spinetoram	Spinosyns (nAChR allosteric modulators/5)	✓	✓	–	✓	–	–
Spinosad	Spinosyns (nAChR allosteric modulators/5)	✓	✓	✓	✓	–	–
Spiromesifen	Tetronic acid derivatives (acetyl CoA carboxylase inhibitors/23)	✓	✓	✓	✓	–	–
Sulfoxaflor	Sulfoximines (nAChR competitive modulators/4C)	✓	✓	–	–	–	–
Teflubenzuron	Benzoylureas (inhibitors of chitin biosynthesis affecting CHS1/15)	✓	✓	✓	✓	–	–
Thiamethoxam	Neonicotinoids (nicotinic acetylcholine receptor competitive modulators/4A)	✓	✓	✓	✓	✓	–

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with multiple modes of action, while Polish farmers are limited to essentially two mechanisms.

Comparative analysis: Poland vs. Mercosur insecticides

The comparative analysis of insecticide availability demonstrates a pronounced asymmetry between Poland and Mercosur countries. Polish soybean production relies on an extremely limited insecticide toolbox, encompassing only two chemical active substances (Tab. 8) and a narrow range of biological agents (Tab. 9), which severely restricts the diversity of modes of action available for pest control. In contrast, Mercosur production systems benefit from broad and functionally diverse chemical and biological insecticide portfolios, enabling rotation of modes of action, stage-specific pest targeting, and integration of chemical

Table 9. Biological insecticide substances for soybean: Poland vs. Mercosur (Source: author's own elaboration based on HOMOLOGA 2025)

Active substance	Type	Poland	Mercosur
Bacillus thuringiensis	bacterium	✓	✓
Beauveria bassiana	entomopathogenic fungus	✓	✓
Sheep fat (repellent)	natural repellent	✓	–
Bacillus firmus	bacterium (nematicide)	–	✓
Paecilomyces lilacinus	entomopathogenic fungus	–	✓
Pasteuria nishizawae	bacterium (nematicide)	–	✓
Argemone mexicana extract	plant extract	–	✓

Table 10. Comparison of Maximum Residue Limits (MRLs) in the EU and Brazil for selected active substances used in soybean

Active substance	EU MRL [mg · kg^{-1}]	Brazil MRL [mg · kg^{-1}]	Ratio
Glyphosate	20	10	EU 2 × higher
2,4-D	0.05	0.1	BR 2 × higher
Chlorothalonil*	0.01	0.5	BR 50 × higher
Mancozeb*	0.1	0.3	BR 3 × higher
Atrazine*	0.01	0.02–0.05	BR 2-5 × higher
Chlorpyrifos*	0.01	2.0	BR 200 × higher
Paraquat*	0.02	0.1	BR 5 × higher
Imidacloprid*	0.05	0.5	BR 10 × higher
Tebuconazole	0.05	0.2	BR 4 × higher
Lambda-cyhalotrin	0.02	0.2	BR 10 × higher
Fipronil*	0.005	0.02–0.05	BR 4-10 × higher
Clomazone	0.05	0.2	BR 4 × higher
Diquat*	0.02	0.05	BR 2.5 × higher

*Active substance not approved in the EU (MRL retained only for imports)

and biological approaches. This disparity substantially constrains the implementation of insecticide resistance management and integrated pest management strategies in Poland, while providing greater flexibility and resilience in Mercosur soybean protection systems.

Maximum Residue Limits: regulatory asymmetries

Maximum Residue Limits established under EU Regulation 396/2005 represent the legal thresholds for pesticide residues in food and feed. Analysis of MRL disparities between the EU and Brazil reveals significant asymmetries with implications for import safety assurance (Barosso *et al*. 2025; Hoffmans *et al*. 2025; Martins and Burnquist 2025). For substances banned in the EU (chlorothalonil, atrazine, chlorpyrifos, imidacloprid, paraquat), the EU applies default MRLs at the limit of analytical detection (typically 0.01 mg/kg), while these substances remain in active use in Brazil with substantially higher MRLs. The 50-fold difference for chlorothalonil and 200-fold difference for chlorpyrifos in citrus illustrate the challenge of ensuring regulatory equivalence when production practices differ fundamentally (Tab. 10).

For substances banned in the EU, default MRLs at the limit of analytical detection apply, whereas higher MRLs are permitted in exporting countries (Regulation EC No 396/2005; OECD 2017, 2021). While MRLs differ substantially between regions, exported products must comply with EU MRL standards; thus, differences do not constitute a direct competitive advantage but may pose challenges for monitoring and enforcement. In this context, the interpretation of MRL disparities has been refined to avoid suggesting a direct competitive advantage for exporting countries. It is important to emphasize that all products placed on the EU market must comply with EU MRL requirements, regardless of the regulatory framework in the country of origin. However, differences in MRL regimes reflect underlying differences in production systems and pesticide use patterns, which may complicate residue monitoring, analytical verification, and enforcement of compliance at the import stage (OECD 2017, 2021; Hoffmans *et al*. 2025). This perspective provides a more balanced and accurate interpretation of the trade implications associated with regulatory asymmetries.

Summary of active substance availability

Table 11 presents a comparative overview of the availability of active substances for soybean plant protection products (PPPs) in Poland and Mercosur countries. The data reveal a pronounced asymmetry in access to

Table 11. Summary comparison of active substance availability for soybean protection: Poland vs. Mercosur (Source: author's own elaboration based on HOMOLOGA 2025)

PPP Category	Poland	Mercosur	Ratio
Herbicides (chemical)	16	96	1 : 6
Fungicides (chemical)	5	96	1 : 19
Fungicides (biological)	2	104	1 : 52
Insecticides (chemical)	2	94	1 : 47
Insecticides (biological)	3	9	1 : 3

crop protection tools between the two regions. Across all PPP categories, producers in Mercosur benefit from a substantially broader portfolio of authorized active substances than those available in Poland. The disparity is particularly acute for biological fungicides, where Mercosur has access to more than 50 times as many active substances as Poland (104 vs. 2). Similarly, large gaps are observed for chemical insecticides (94 vs. 2; ratio 1 : 47) and chemical fungicides (96 vs. 5; ratio 1 : 19), indicating markedly different levels of regulatory permissiveness and technological availability.

The biological control paradox

The 52 : 1 disparity in biological fungicide strains between Mercosur (104) and Poland (2) presents a striking paradox. The European Union has explicitly prioritized biological alternatives through the Sustainable Use Directive, Green Deal, and Farm to Fork Strategy, yet Mercosur countries – particularly Brazil Insect growth regulators – have achieved far greater diversification of biological control options. This counterintuitive finding merits careful analysis.

Several factors contribute to this paradox. First, Brazil's regulatory system treats each microbial strain as a distinct active substance, enabling rapid expansion of registered options. Second, the scale of Brazilian agriculture creates commercial incentives for biological product development unavailable in smaller markets. Third, the year-round pest pressure in tropical systems drives continuous demand for alternative control methods when chemical options face resistance problems. Fourth, the structure of Mercosur agricultural research – with strong linkages between Embrapa, state research institutions, and commercial developers – accelerates translation from laboratory to field.

It should also be noted that the limited number of biological active substances registered in Poland (currently only two microbial strains) are generally compatible with organic farming systems and align with sustainability objectives. However, their extremely low diversity significantly constrains their practical applicability in both conventional and organic soybean production. This limitation reduces flexibility in disease management, restricts the possibility of combining or rotating biological agents, and ultimately diminishes the effectiveness of biological control strategies under field conditions.

The implications for EU policy are significant. Despite regulatory frameworks designed to promote biological alternatives, practical implementation has not delivered the diversification achieved in Mercosur. Previous studies have identified regulatory complexity, lengthy approval procedures, and high data requirements as key bottlenecks limiting the development and adoption of biological plant protection products in the EU (Balog *et al*. 2017; van Lenteren *et al*. 2018). Accelerating EU registration procedures for biological PPPs, perhaps through mutual recognition agreements or expedited review pathways, could address this gap while advancing sustainability objectives.

Implications for the EU–Mercosur Partnership Agreement

The regulatory asymmetries documented in this study have profound implications for the EU-Mercosur Partnership Agreement finalized in December 2024:

Competitive disadvantage: European soybean producers operate with a fraction of the crop protection tools available to Mercosur counterparts. The 6-fold to 52-fold differences in active substance availability translate into higher production costs, reduced yield potential, and limited capacity to respond to emerging pest and disease pressures. Under tariff liberalization, this asymmetry may accelerate the structural decline of European protein crop production.

Food safety concerns: Approximately 100 active substances used in Mercosur soybean production are not approved for use in the EU, with many explicitly banned due to health or environmental concerns. While imported products must comply with EU Maximum Residue Limits, the use of substances with MRLs 50–200 times higher in Brazil raises questions about effective import surveillance and enforcement capacity.

Structural dependency: The EU imports over 50% of its soybean and soybean meal requirements from Mercosur countries, creating a fundamental dependency with limited short-term alternatives. This dependency constrains the EU's ability to impose stringent import standards without risking supply disruption to the livestock sector.

Regulatory coherence: The simultaneous pursuit of pesticide reduction under the Green Deal and trade liberalization with regions maintaining broader pesticide portfolios creates internal policy tension. Addressing this tension requires either strengthened import controls (potentially facing World Trade Organization (WTO) challenges) or acceptance of asymmetric competitive conditions.

Discussion, conclusions and recommendations

This study demonstrates that regulatory differences in the authorization of plant protection products translate directly into asymmetries in pest management capacity between the European Union and Mercosur countries (Ambroziak *et al*. 2025). From a plant protection

perspective, the observed disparities in the number of authorized active substances, diversity of modes of action, and availability of biological control agents have practical implications for resistance management, yield stability, and production resilience in soybean cultivation.

In the European Union, the progressive reduction in authorized chemical active substances has narrowed the spectrum of available tools for soybean protection. While this approach reflects precautionary regulatory objectives, it also constrains the practical implementation of integrated pest management, particularly where alternative modes of action are limited. The restricted availability of herbicides and insecticides increases the risk of resistance development, a phenomenon already documented for several key weed and insect species in European arable systems. This is consistent with broader evidence indicating that reduced diversity of modes of action is a primary driver of resistance evolution, particularly in simplified production systems (Norsworthy *et al*. 2012; Hicks *et al*. 2018; Hawkins *et al*. 2019).

By contrast, Mercosur production systems operate with substantially broader plant protection portfolios, allowing for diversified control strategies and systematic rotation of modes of action. This is particularly relevant for the management of Asian soybean rust, where intensive fungicide programs and access to multiple chemical classes are essential to delay resistance development. The extensive registration of biological fungicides at strain level further enhances the flexibility of disease management strategies in South American soybean production.

The pronounced disparity in biological control availability represents a regulatory bottleneck rather than a technological limitation in the EU context. Despite strong policy support for biological plant protection products, the limited number of authorized microbial strains constrains their practical use in soybean cultivation (Bueno *et al*. 2023). Accelerated and harmonized registration pathways for low-risk biological agents could therefore play a key role in strengthening EU plant protection systems while remaining consistent with sustainability objectives.

Overall, the findings indicate that plant protection considerations should be more explicitly integrated into assessments of trade agreements involving agricultural commodities. Regulatory asymmetries in pesticide availability not only affect competitiveness but also shape phytosanitary risk management and long-term sustainability of crop production systems.

Recent policy developments at the EU level further reinforce the relevance of these findings. The Food and Feed Omnibus package adopted in December 2025 introduces measures aimed at accelerating approval procedures for biological plant protection products, thereby addressing one of the key regulatory bottlenecks identified in this study. These changes are closely aligned with broader EU strategic objectives, including the European Green Deal and the Farm to Fork Strategy, which prioritize the reduction of chemical pesticide use and the promotion of sustainable alternatives. If effectively implemented, the revised regulatory framework may contribute to reducing the current disparities in the availability of biological control solutions between the EU and Mercosur countries, strengthening integrated pest management capacity while maintaining high standards of environmental and human health protection.

This comparative analysis demonstrates that substantial regulatory asymmetries exist between the European Union and Mercosur countries in the availability of plant protection products for soybean cultivation. These differences extend across all major categories of crop protection, including herbicides, fungicides, insecticides, and biological control agents, and they directly influence pest management capacity and resistance risk.

In Brazil, the approval status of pesticide-active ingredients is very discrepant compared to other agricultural countries. Moreover, the duality of benefits and risks of pesticide application creates an economic and toxicological conflict (Souza *et al.* 2023). From a plant protection perspective, the limited spectrum of authorized active substances in the EU constrains the implementation of diversified and resistance-aware management strategies. In soybean production, where effective control of weeds, fungal diseases, and insect pests depends on access to multiple modes of action, such constraints may reduce production resilience and increase vulnerability to emerging phytosanitary threats.

The policy implications of these findings have been further expanded in light of the ongoing European debate on trade and regulatory alignment. In particular, increasing attention is being paid to the concept of "mirror clauses", which would require imported agricultural products to comply with standards equivalent to those applied within the EU. In parallel, discussions include the potential lowering of MRLs for non-approved substances to the level of technical zero, as well as strengthening border control systems, for example through a substantial increase in inspection frequency (e.g., by 50%). These proposals are actively debated at the level of the European Commission and Member States, including countries such as France, reflecting growing political pressure to ensure consistency between domestic regulatory standards and import requirements. Taken together, these developments indicate that regulatory asymmetries in plant protection are becoming a central issue in EU trade policy, with potential implications for market access, compliance costs, and the future structure of agri-food supply chains.

The practical consequences of these regulatory constraints are, however, context-dependent and vary across pest groups and management scenarios. In some cases, effective control remains feasible despite a limited spectrum of authorized substances. For example, weed management in soybean can still be maintained under moderate pressure through reliance on a reduced set of herbicides, such as ACCase inhibitors, when complemented by appropriate agronomic practices including crop rotation, mechanical control, and optimization of sowing systems. In contrast, other situations represent critical limitations, particularly where effective control depends on access to multiple modes of action. This is exemplified by the management of Asian soybean rust (*Phakopsora pachyrhizi*), a highly destructive disease requiring intensive fungicide programs and systematic rotation of active ingredients to delay resistance development. The restricted fungicide portfolio available in the EU significantly constrains both resistance management and disease control capacity in this context. These contrasting examples illustrate that the implications of regulatory restrictions are not uniform, but range from manageable to highly limiting depending on the biological characteristics of the target organism and the availability of complementary management strategies.

The markedly broader availability of biological fungicides in Mercosur countries highlights that regulatory processes, rather than technological limitations, are a key factor shaping the practical adoption of biological control (OECD 2017, 2021; EFSA, multiple years). Streamlining authorization procedures for low-risk biological agents could, therefore, strengthen integrated pest management in the EU while remaining consistent with sustainability objectives.

Overall, the findings underline the importance of explicitly considering plant protection systems in the context of expanding international trade in agricultural commodities. Future research should focus on the long-term implications of regulatory asymmetries for resistance evolution, crop health, and the sustainability of soybean production systems under increasingly interconnected global markets, with particular emphasis on resistance dynamics and Integrated Pest Management (IPM) robustness (Cerdeira *et al*. 2011; Heap 2025; FRAC 2023; IRAC 2023).

This comprehensive analysis reveals fundamental structural asymmetries in crop protection systems between the European Union and Mercosur that have significant implications for agricultural trade, food safety, and production sustainability. The documented disparities – ranging from 6 : 1 for herbicides to 52 : 1 for biological fungicides – represent not merely quantitative differences but fundamentally different regulatory philosophies and production paradigms. Importantly, the observed regulatory asymmetries should also be interpreted in light of the underlying risk rationale guiding pesticide authorization decisions in the European Union. A substantial proportion of active substances no longer approved in the EU have been withdrawn due to well-documented concerns related to human health (including carcinogenicity and endocrine disruption), environmental persistence and bioaccumulation, as well as toxicity to non-target organisms such as pollinators and aquatic species. This reflects the fundamentally hazard-based nature of the EU regulatory framework, in contrast to the predominantly risk-based approaches applied in Mercosur countries. This distinction has been widely discussed in the literature on EU risk governance, where the precautionary principle plays a central role in regulatory decision-making (European Commission 2020), particularly in the context of plant protection product risk assessment frameworks. While this precautionary paradigm contributes to higher levels of health and environmental protection, it simultaneously reduces the diversity of available modes of action, thereby influencing pest management flexibility and resistance dynamics. Consequently, the differences identified in this study should not be interpreted solely as disparities in regulatory efficiency or market access, but rather as outcomes of distinct regulatory philosophies that balance agricultural productivity, environmental protection, and public health in different ways. This perspective enhances the policy relevance of the present analysis, particularly in the context of ongoing evolution of EU pesticide legislation and its implications for international trade.

Based on these findings, we propose the following policy recommendations:

Enforceable mirror clauses: The EU should negotiate enforceable provisions requiring that imported agricultural products meet equivalent production standards, particularly regarding the use of active substances banned in the EU. While such provisions face WTO scrutiny, they represent the most direct approach to ensuring regulatory coherence.

Enhanced import surveillance: Expanded residue testing programs should specifically target substances banned in the EU but permitted in exporting countries. Current sampling rates may be insufficient to ensure compliance given the scale of Mercosur imports and the breadth of substances in use.

Compensatory mechanisms: Recognition that EU producers face structurally higher costs due to regulatory restrictions should inform support mechanisms under the Common Agricultural Policy, potentially including enhanced direct payments for protein crop production.

Accelerated biological PPP registration: The paradox of Mercosur's greater biological control diversification should prompt review of EU registration procedures. Expedited pathways for low-risk biological products could advance sustainability objectives while expanding the crop protection toolkit.

Strategic protein security investment: Reducing structural dependency on Mercosur soybean imports requires long-term investment in European protein crop production, including dedicated research programs, breeding initiatives, and market development support.

Continuous monitoring: Establishment of systematic market monitoring mechanisms to track the evolution of competitive conditions post-agreement, with defined safeguard activation thresholds.

The EU–Mercosur Partnership Agreement represents a historic development in global agricultural trade. Ensuring that this agreement delivers mutual benefits while protecting European production standards, food safety, and environmental sustainability requires clear-eyed assessment of the asymmetries documented in this study and policy responses adequate to address them.

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ORIGINAL ARTICLE

Antifeedant and insecticidal activity of four medicinal plants from arid regions on adults of the red flour beetle *Tribolium castaneum* (*Coleoptera, Tenebrionidae*)

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Abstract

Considering the importance of sustainable pest control, this study evaluated the insecticidal and antifeedant properties of medicinal plants from arid regions against *Tribolium castaneum*. The methodology involved extracting bioactive compounds from four selected plant species: *Atriplex halimus* L. (saltbush), *Sonchus oleraceus* L. (sowthistle), *Lavandula multifida* L. (fernleaf lavender) and *Globularia alypum* L. (globe daisy), and conducting antifeedant and insecticidal bioassays, along with phytochemical screening tests, to assess their effects on adult beetles. The primary findings indicated that *Lavandula multifida* and *Sonchus oleraceus* manifested notable insecticidal activity, underscoring their promise as efficacious natural pest management agents, despite their modest antifeedant activities. Notably, Atriplex halimus demonstrated remarkable antifeedant activity, achieving a 31.37% effect, though its insecticidal activity was somewhat modest. Globularia alypum showed minimal insecticidal and antifeedant activity. However, the utilization of Lavandula multifida as a bioinsecticide may be limited by the plant's small size, which necessitates the production of a considerable quantity of plant material to produce a sufficient extract. Phytochemical analysis indicated that these plants are rich in bioactive compounds, including flavonoids, terpenoids, and tannins. The observed differences between insecticidal and antifeedant activities suggest the presence of different underlying mechanisms. This study underscores the potential of these plant extracts as environmentally friendly alternatives to synthetic pesticides and lays the groundwork for future research into their role in sustainable pest management strategies.

Key words: antifeedant activity, arid regions, insecticidal activity, medicinal plants, sustainable pest control, *Tribolium castaneum*

Introduction

Insect pests pose a considerable menace to global food security, with the greatest risk to agricultural ecosystems. These pests inflict considerable damage to crops and stored food products, resulting in significant disruptions to global food production and availability. The primary damage caused by insects to stored foodstuffs is through direct consumption (Manandhar *et al.* 2018). Also, they are vectors for fungal infections, which can increase the risk of contamination with mycotoxins and reduce the quality of stored

grains (Eigenbrode *et al.* 2018; Yun *et al.* 2018). The red flour beetle, *Tribolium castaneum* (Herbst 1797) (Coleoptera: Tenebrionidae), is of particular concern because it is a common stored grain pest, affecting processed cereals, oilseeds, nuts, and dried fruits. Despite the implementation of various preventive and control measures, *T. castaneum* continues to pose a persistent problem in storage depots (Swamy *et al.* 2020). Furthermore, this species has developed resistance to chemical insecticides, which makes its management more complex (Astuti *et al.* 2020).

Approximately, two million tons of pesticides are applied globally each year for insect damage control (Sharma *et al.* 2019). However, the massive use of chemical pesticides has serious consequences for the environment and human health. These substances are toxic, accumulate in the environment, and resist degradation, which leads to their prolonged presence in ecosystems. In addition, unreasonable pesticide use has led to a number of complications, including the development of insecticide resistance, the appearance of secondary pests, damage to beneficial insects, and the presence of pesticide residues in food products (Bass *et al.* 2014; Pathak *et al.* 2022). It is recommended that pest control methods prioritize environmental and ecological considerations, giving preference to the use of biological and physical control methods over other strategies. Chemical treatments should be used only as a last resort. Despite the speed and cost-effectiveness of chemical methods, which involve the use of insecticides in stored produce, they also present risks, including grain contamination and toxicity to humans and animals (Arora *et al.* 2021).

Scientific research has recently shown interest in the potential use of medicinal plants as a source of bioactive compounds with insecticidal properties. The field of ethnopharmacology has strongly captured the attention of the pharmaceutical and medical communities due to the growing recognition of the potential of these natural compounds, which are often unlikely to have adverse effects (Gacem *et al.* 2019). In this context, medicinal plants from arid and semi-arid regions, such as those found in Algeria, where these climates cover over 87% of the land surface, are of particular interest. Despite the harsh environmental conditions, these regions are home to over 2,800 plant species, many of which have unique phytochemical profiles and have been traditionally used in medicine (Halla *et al.* 2018; Behaz *et al.* 2024).

The bioactive molecules present in these plants often have complementary or synergistic therapeutic effects, and their potential as natural insecticides is increasingly recognized. Furthermore, the antioxidant properties of these plants reinforce their value in combating pests and maintaining food quality and safety (Chaouche *et al.* 2020; Haddouchi *et al.* 2021).

Despite the numerous studies about the bioactivity of medicinal plants, there is still limited information about their application in agricultural sciences, specifically in the protection of stored food products against insect pests. Plant-derived products, including crude extracts, have promise as biological alternatives for controlling storage pests like *T. castaneum* (Herbest) reducing chemical residues in food products, and minimizing environmental impact. However, research on plants from arid and semi-arid regions remains limited, particularly concerning their insecticidal and antifeedant effects under storage conditions.

In this context, the present study aimed to evaluate the insecticidal and antifeedant effects of four medicinal plants: *Atriplex halimus* L. (Mediterranean saltbush; Amaranthaceae), *Sonchus oleraceus* L. (Common sowthistle; Asteraceae), *Lavandula multifida* L. (Fernleaf lavender; Lamiaceae), and *Globularia alypum* L. (Globe daisy; Plantaginaceae), which are found in the arid regions of Algeria, against *Tribolium castaneum* (Herbest) a major stored-product pest. The goal was to identify natural alternatives for managing insect infestations in storage environments, contributing to food preservation and environmentally friendly pest control practices.

Materials and Methods

Insect breeding

Colonies of *T. castaneum* were reared in glass jars containing semolina as a substrate. The jars, covered with fine mesh for ventilation, were placed in an incubator at 30 ± 3°C and about 70% relative humidity. Breeding took place at the Laboratory of Ecosystems Diversity and Agricultural Production Systems Dynamics in Arid Zones (DEDSPAZA) located at the University of Biskra. This species was selected due to its significant economic impact on stored products and for its suitability for mass rearing under laboratory conditions, allowing for the collection of large numbers of individuals for bioassays.

Plant material

The aerial parts of four plant species, *A. halimus* L., *S. oleraceus* L., *L. multifida* L., and *G. alypum* L., were collected during the spring season from various arid regions in Algeria (Table 1). The collected specimens were identified and taxonomically authenticated by Dr. Abdelkader Nabil Benghanem, lecturer and researcher in botany at the National Higher School of Agronomy, Algiers. Identification was confirmed using standard botanical keys and regional floras, including Nouvelle Flore de l'Algérie et des Régions Désertiques

Table 1. Botanical identity, common names, and collection site details of the studied plants

Family	Species	Common name	Bioclimatic floor, geographic coordinates
Amaranthaceae	*Atriplex halimus* L	mediterranean saltbush	Arid, 34°45′33″N, 5°53′34″E
Asteraceae	*Sonchus oleraceus* L	common sowthistle	Arid, 34°45′32″N, 5°53′40″E
Lamiaceae	*Lavandula multifida* L	fernleaf lavender	Arid, 34°56′10″N, 5°59′08″E
Plantaginaceae	*Globularia alypum* L	globe daisy	Arid, 34°59′47.3″N, 6°3′0.75″E

Méridionales (Quézel and Santa 1962, 1963) and Flore d'Afrique du Nord Maire (1964).

The plant materials were air-dried for three to four weeks in a well-ventilated area, and away from direct light from direct light. Each dried sample was ground into a fine powder using an electric grinder and stored at room temperature until further use.

Preparation of the extract

For extraction, 50 g of each powder was mixed with 500 ml of ethanol solvent, and 100 ml of distilled water was added to an Erlenmeyer flask covered with aluminum foil. The mixture was stirred for two hours using a magnetic stirrer. During this step, it was ensured that the mixture was homogeneously agitated before proceeding to maceration. The solutions were macerated in the shade at room temperature for 48 hours. After filtration, each extracted solution underwent pressure evaporation at 45°C in a rotary evaporator to eliminate a significant amount of the solvent. The resultant plant concentrates were then kept in glass vials, safeguarded from light at 4°C for future use.

Phytochemical screening analysis

Phytochemical screening was carried out using qualitative methods as described by Shaikh and Patil (2020). The following tests were performed to detect various phytochemicals:

- **Alkaloids** were detected by using the tannic acid test. Buff precipitate indicates their presence;
- **Flavonoids** were detected by using the alkaline reagent test. The appearance of an intense yellow color that disappears with HCl confirms the presence of flavonoids;
- **Total polyphenols** were identified using the potassium dichromate test. A dark color indicates their presence;
- **Tannins** were detected by using the test; emulsion formation indicates the presence of hydrolysable tannins;
- **Saponins** were detected using the foam test. The appearance of foam that persists more than 10 minutes means there are saponins in the extract;
- **Terpenoids** were detected by testing with chloroform and concentrated H_2SO_4. Their presence was indicated with the formation of a dense golden--yellow layer;
- **Quinones** were detected with the concentrated HCl test. A green color indicates the presence of quinones;
- **Coumarins** were determined by using the NaOH test (10% NaOH). The appearence of yellow color confirms coumarins;
- **Anthraquinones** were detected with the Borntrager's test. After shaking, a violet to red solution indicates their presence.

Toxicity tests (contact/inhalation)

For each plant, the crude extract obtained through solvent extraction was used in these tests. Different doses (D1 = 10%, D2 = 30%, and D3 = 50%) were applied to the surface of Petri dishes containing 10 adults of *T. castaneum* and 2 grams of wheat semolina. Each dose was repeated three times, and the control was treated with distilled water only. The dishes were preserved under the same rearing conditions, and dead individuals were counted every day for 21 days.

Antifeedant test

The evaluation of antifeedant activity and the applied protocol were inspired by methodologies commonly used in recent studies on insect–plant interactions (Mayanglambam et *al.* 2022; Ajaha *et al.* 2023; Tine *et al.* 2023).

Prepared disks containing 2 g of semolina, treated with 5 µl of plant extract (treated group with 30% dose) or distilled water (control group), were weighed after a brief evaporation period. Ten adult *T. castaneum* insects, fasted for 2 hours, were introduced to each disk. Each test was conducted with three replications (Fig. 1).

Choice Test

In this test, insects were given a choice between 2 g of treated semolina and 2 g of untreated semolina (Fig. 1).

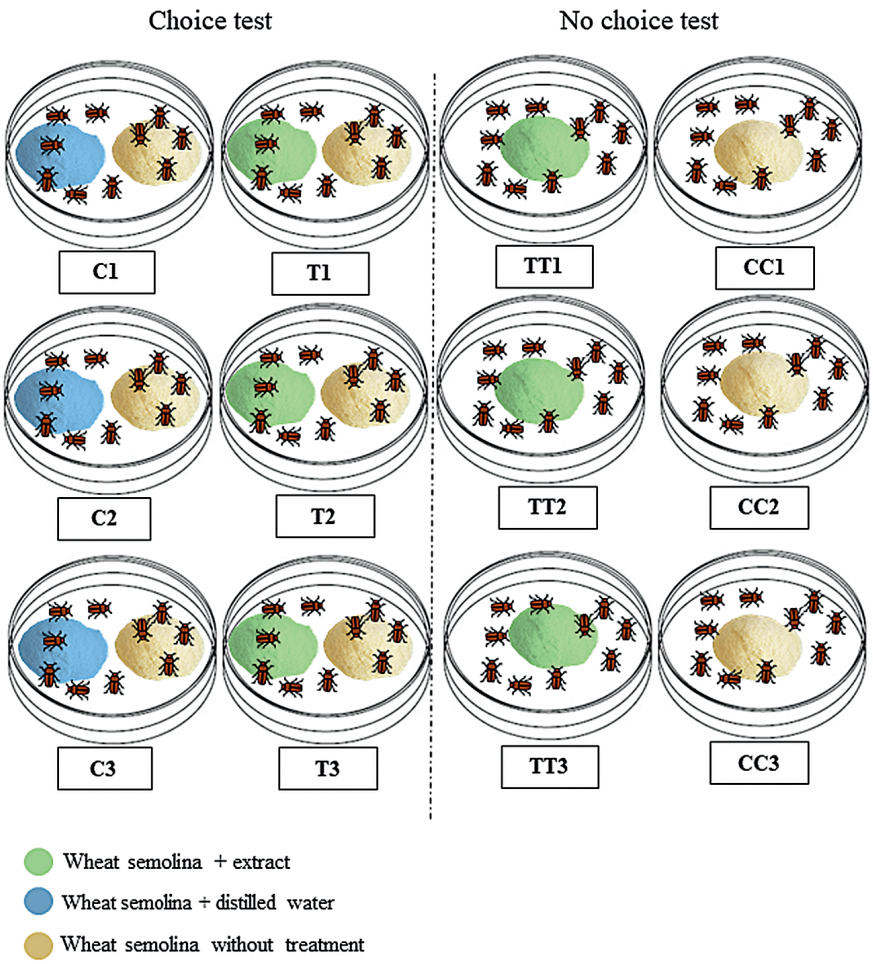

Fig. 1. Antifeedant test: choice and no choice test protocol (Original 2024)

No-Choice Test

In this test, insects were presented with a single 2 g semolina disk, either treated with the plant extract or untreated, with three replications for each condition (Fig. 1).

The weight of the semolina in each test was recorded daily over 7 days. Food consumption by the insects was calculated by subtracting the weight recorded each day from that of the previous day.

- Discs without treatment (CC).
- Discs where there was a choice between a treated disc (with distilled water) and a non-treated disc (C).
- Treated discs where there was no choice (TT).
- Discs where there was a choice between a treated disc (with extract) and a non-treated disc (T).

The use of the formula by Nawrot *et al.* (1986):

Choice Test

$$\%R = \frac{C - T}{C + T} \times 100,$$

where:
C – weight of consumed semolina for the control group (mg);
T – weight of consumed semolina for the treated group (mg);
R – relative antifeedant activity (%).

No-choice Test

$$\%A = \frac{CC - TT}{CC + TT} \times 100,$$

where:
CC – weight of consumed semolina for the control group (mg);
TT – weight of semolina for the treated group (mg);
A – absolute antifeedant activity (%).

These equations serve to quantify the anti-appetence activity of the treatments in comparison to the control group, both in a choice test and in a no-choice test.
The total:

$$T = A + R,$$

where:
T – total inhibition coefficient value;
A – absolute anti-appetence activity (%);
R – relative anti-appetence activity (%).

The categories of anti-appetence activity based on Gabrys *et al.* (2006) are as follows:

Table 2. Groups of antifeedant activity based on the total value of the inhibition coefficient

N°	T value	Category of antifeedant activity
1	< 50	weak deterrents
2	51–100	medium deterrents
3	101–150	good deterrents
4	151–200	very good deterrents
5	negative	attractant properties

Data analysis

Mortality correction

Mortality rates were corrected using Schneider-Orelli's (1947) formula

$$\text{Corrected mortality (CM) (\%)} = \frac{\text{Mt (\%)} - \text{M0 (\%)}}{100 - \text{M0 (\%)}} \times 100,$$

where:
CM – Corrected mortality (%);
Mt – Mortality of treated sample (%);
M0 – Mortality of untreated sample (%).

Regression lines were constructed by linking the corrected mortality rates expressed in Probits to the extract doses.

Statistical analysis

All results are presented as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics version 26. One-way ANOVA followed by chi-square test was used to compare differences between groups. Probit analysis was applied to calculate lethal doses (LD_{50} and LD_{90}) with 95% confidence limits. Differences were considered statistically significant at $p < 0.05$.

Results

Toxicity effect of plant extracts

The results showed an increase in the percentage mortality of *T. castaneum* adults as a function of time and plant extract dose (Fig. 2). In general, no mortality was observed in the control groups during

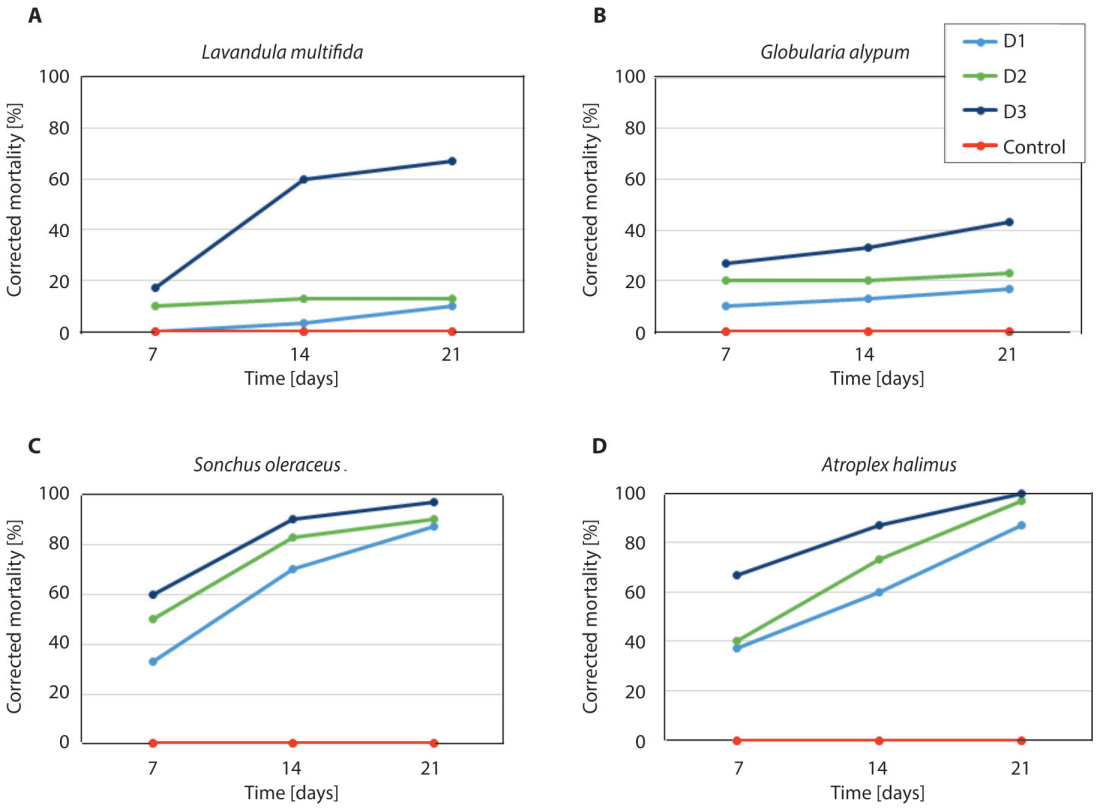

Fig. 2. Percentage change in corrected mortality of *Tribolium castaneum* adults as a function of time and the dose of the studied Saharan plant extracts

the exposure period. As illustrated in Figure 2B, for *G. alypum*, mortality increased with dose, reaching approximately 40% after 21 days for D3, in comparison to the dose of 30% (D2), and 10% (D1), respectively (around 10 to 20%). The effects observed for *L. multifida* (Fig. 2A) were more moderate, with maximum mortality of approximately 60% for D3, while D2 and D1 showed lower rates, at around 20%. In contrast, *A. halimus* (Fig. 2D) and *S. oleraceus* (Fig. 2C) exhibited a more rapid and significant effect, with mortality rates exceeding around 70 to 85% as early as 14 days for D2 and D3, reaching approximately 100% at 21 days. In comparison, D1 demonstrated less efficacy, with mortality rates ranging between 60% and 65% in 14 days. Based on results in Figure 2, comparing the mortality in the highest dose D3, the results showed that *A. halimus* extract exhibited the highest mortality percentage, ranging from 67% to 100%, with highly significant P-values (<0.001) (Table 3). This was followed by *S. oleraceus* extract (60% to 97%), *L. multifida* extract (17% to 67%), and *Globularia alypum* extract (27% to 43%) (Fig. 2).

The LD50, LD90, LT50, and LT90 values represent the lethal doses and times for extracts from four plants (*G. alypum, L. multifida, A. halimus*, and *S. oleraceus*) (Table 3).

The efficacy of *S. oleraceus* was consistently the highest, requiring the lowest doses and shortest times to achieve significant mortality rates. This was indicated by the LD50 (3.76 mg · ml^{-1}), LT50 (2.91 hours), and LT90 (6.89 hours) values. Furthermore, the LT50 (2.91 hours) and LT90 (6.89 hours) values are noteworthy (Table 3). This suggests that *S. oleraceus* contained bioactive compounds that were both rapid and effective in their action against target pests. In contrast, *L. multifida* was the least effective, with the highest LT90 (35.95 hours) (Table 3), indicating the necessity for higher concentrations and longer exposure times to achieve comparable results. This may restrict its practical application without further improvement. The efficacy of *A. halimus* and *G. alypum* was moderate, with *A. halimus* demonstrating greater efficacy at lower doses, while *G. alypum* required the longest time for complete pest mortality (LT90: 445.29 hours) (Table 3).

Overall, *S. oleraceus* was the most promising candidate for developing rapid and efficient plant-based insecticides, while *L. multifida* may require further refinement to enhance its effectiveness. The moderate efficacy of *A. halimus* and *G. alypum* suggests potential for use in specific contexts, depending on the required balance between dose and action time.

The extremely low *P*-values (<0.05) across all lethal doses and times suggest that there are highly significant differences between the effects of the four plant extracts (Table 3).

Antifeedant activity

All extracts provided low antifeedant activity, with indices below 50% (Fig. 3). The *G. alypum* extract showed minimal activity with an index value of 2%. However, *L. multifida* and *S. oleraceus* extracts were slightly attractive, with negative index values of –3% and –0.5%, respectively. The highest antifeedant activity was observed in *A. halimus* with a value of 31.37%.

A chi-square test was performed and revealed a statistically significant association between antifeedant indices and the extracts studied ($\chi^2 = 68.405$, df = 3, $p < 0.001$). The high value of the chi-square statistic and the very low probability suggest that the differences observed were not due to chance. No contingency cell had a theoretical frequency of less than five, confirming the reliability of the results. This association indicates that plant type had a significant influence on antifeedant indices, which should be taken into consideration when managing insect populations.

Preliminary phytochemical screening

Phytochemical screening of the four medicinal plants (*L. multifida, G. alypum, A. halimus and S. oleraceus*) revealed the presence of various bioactive compounds including flavonoids, phenolic compounds, terpenoids and alkaloids known for their insecticidal and antifeedant properties (Table 4). The consistent presence

Table. 3. Lethal dose and time analysis for selected plant extracts

Measure [mg · ml^{-1}]	Plant extracts				Average ± Sd	Variation coefficient	*P*- value
	G. alypum	*L. multifida*	*A. halimus*	*S. oleraceus*			
LD50	7.71	18.77	13.81	3.76	11.01 ± 6.62	0.60	.000
LD90	60.77	48.14	31.33	30.11	42.59 ±14.65	0.34	.000
LT50	23.93	13.59	6.40	2.91	11.71 ± 9.28	0.79	.000
LT90	445.29	35.95	11.27	6.89	124.85 ± 214.01	1.71	.000

LD and LT values are presented with their corresponding 95% confidence limits (CL)

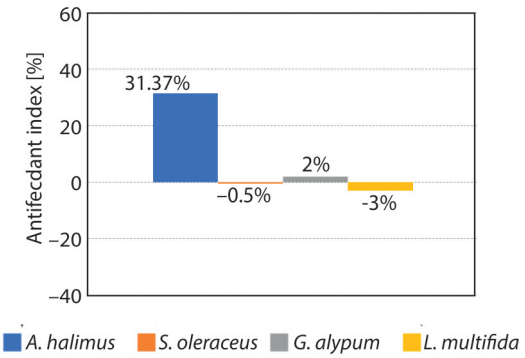

Fig. 3 Antifeedant effects of four different medicinal plant extracts against *Tribolium castaneum* adults

Table 4. Preliminary phytochemical screening results of tested plant extracts

Phytochemical classe	*L. multifida*	*G. alypum*	*A. halimus*	*S. oleraceus*
Alkaloids	–	++++	+	+
Flavonoids	+++	+++	+	++++
Phenolic compounds	+++	++++	++	++
Tannins	+++	++	+	++
Saponins	+	+++	++	–
Terpinoides	+	++++	++	++++
Quinones	++	+++	+++	+
Couramins	++++	++++	++	+
Anthraquinones	++++	+++	++++	++

Very hight – (++++), hight – (+++), moderate – (++), low – (+) and absent – (–)

of these compounds in all plants suggests a potential synergy in their bioactivity against *T. castaneum*. Notably, *A. halimus*, which showed the most significant antifeedant activity (31.37%), was rich in several phytochemicals, such as flavonoids and terpenoids, which may contribute to its strong deterrent effect. Conversely, the weaker antifeedant effects observed in *L. multifida* and *S. oleraceus* (with negative or minimal activity) could be related to the lower or absent concentrations of certain key compounds such as alkaloids.

However, the preliminary screening results indicate that flavonoids, phenolic compounds, and tannins were the predominant phytochemical groups in most of the tested plants. These groups are commonly associated with biological activities such as antioxidant, antimicrobial, and insecticidal effects. Their relative abundance in *G. alypum* and *L. multifida* suggests that they may play a significant role in the observed insecticidal properties. Further detailed chemical analysis would be necessary to identify the specific active compounds responsible for these effects.

The intensity of phytochemical presence was determined using a semi-quantitative scale [(–) – absent, (+) – present in low amount, (++) – moderate, (+++) – high, (++++) – very high], following the visual evaluation method described in previous works (Haddouchi *et al.* 2021; Madike *et al.* 2017).

Discussion

Naboulsi *et al.* (2022) demonstrated that the aqueous extract of *Atriplex halimus* exhibited strong insecticidal activity against *Dactylopius opuntiae Cockerell* (*Dactylopiidae*). This effect was attributed to the high content of triterpenoid saponins according to their LC-MS analysis. Although LC-MS was not carried out in the present study, phytochemical screening confirmed the presence of these compounds, as the bioactive constituents of *A. halimus*, which could contribute to the insecticidal effects observed in these experiments against *T. castaneum*. Similarly, the aqueous extract of *Carica papaya L.* (*Caricaceae*) leaves showed a notable repellent effect against *T. castaneum*, with efficacy rates ranging from 82% to 97%. This effect was attributed to the presence of 2-methoxy-4-vinylphenol (Mangang *et al.* 2020).

Aissani *et al.* (2022) indicated that *S. oleraceus* L. is a rich source of various phytochemicals, such as polyphenols, flavonoids, sterols, triterpenes, and anthraquinone glycosides. These compounds exist in both hydro-methanolic and hot aqueous extracts. This phytochemical profile suggests that this plant may contain important bioactive properties, consistent with the insecticidal activity observed against *T. castaneum* (El-Kamali 2009). The same author, El-Kamali (2009), confirmed that the alcoholic extract of *S. oleraceus* had a high insecticidal effect on *T. castaneum*, with an LC50 value of 20 mg · ml^{-1} after 36 hours. This validates the efficacy of plant extracts in pest control. Furthermore, *S. oleraceus* leaf extracts have shown a certain degree of repellency against cotton mealybugs in the laboratory (Roonjho *et al.* 2013). Although this repellent activity has not been directly tested on *T. castaneum*, it reveals the potential of *S. oleraceus* extracts as a tool for controlling pests, including stored product insects such as *T. castaneum*.

Lavandula multifida revealed significant insecticidal properties. The LC50 value of its essential oil against *Spodoptera littoralis Boisduval (Noctuidae)* was 2.350 mg · ml^{-1}, while it reached 2.91 mg · ml^{-1} 96 hours after treatment for *Agrotis ipsilon* Hufnagel (Noctuidae) larvae. These results, along with the shorter development periods observed in *S. littoralis* larvae and pupae, suggest that *L. multifida* could serve as an effective pest control agent. However, these findings contrast with the absence of a repellent effect of *L. mairei var.*

antiatlantica essential oil against *T. castaneum* (Kanda *et al.* 2017), highlighting the variability of efficacy between different *Lavandula* species.

In comparison, essential oils of *Eucalyptus globulus* Labill. (Myrtaceae) and *Lavandula stoechas* L. (Lamiaceae) showed significant antifeedant effects on *T. castaneum* adults, suggesting that essential oils extracted from different plant species can be used as effective deterrents against this pest (Ebadollahi 2011). However, Kanda *et al.* (2017) reported that the essential oil of *L. mairei var. antiatlantica* showed no deterrent effect on *T. castaneum*, underscoring that the efficacy depends on the plant species and its specific composition. These results align with those observed in the present study. The coefficient of variation further highlights the consistency of LD50 and LD90 values, while differences observed in LT50 and LT90 values suggest that the time required to achieve 50% and 90% mortality may be influenced by other environmental or biological factors.

G. alypum leaves contain high concentrations of polyphenols and flavonoids. Although the extracts showed antioxidant activity, this was less intense than that of the positive control, ascorbic acid (Yahya *et al.* 2023). This suggests that *G. alypum* may possess additional bioactive compounds, although further research is required to determine its insecticidal and repellent activities. Additionally, the elevated mean LT90 observed for *G. alypum* indicates that, despite its rich phytochemical composition, its insecticidal activity may act very slowly, highlighting the complex interactions between phytochemicals and insecticidal efficacy.

Chi-squared test results indicated a significant relationship between plant antifeedant indices and insect feeding behavior.

In the present study, *A. halimus* showed the highest antifeedant index (31.37%), suggesting some deterrent effect. This could be linked to its phytochemical richness, especially phenolic compounds. In contrast, *G. alypum* showed a very low antifeedant index (2%), while *S. oleraceus* and *L. multifida* exhibited negative values (−0.5% and −3%, respectively), indicating slight attractiveness. These variations can be explained by differences in the type and concentration of secondary metabolites, confirming that not all bioactive compounds exert antifeedant effects in the same way. Futhermore, the applied dose and quantity may have an effect. More specific tests are recommended to determine the chemical compounds and their concentrations in the different plant extracts.

This suggests that plant species and the extract doses choice play an important role in regulating plant-insect interactions and acting as a natural deterrent against pests. The absence of theoretical frequency cells of less than five in the chi-square test further validates these results, giving a strong interpretation to the observed results. This could influence insect population management strategies by encouraging the use of specific natural plant species to minimize pest impact and reduce the use of chemical pesticides in crop protection.

These results indicate the possibility of integrating plants' natural antifeedant properties in crop management strategies, serving to promote more sustainable, eco-friendly agricultural practices. However, a direct correlation between antifeedant activity and insecticidal efficacy has yet to be demonstrated, highlighting the necessity for further research in this field.

In summary, the literature supports the efficacy of certain plant extracts, including those derived from *S. oleraceus*, *A. halimus*, and *L. multifida*, in managing insect pests such as *T. castaneum*. However, consistent with the results obtained in the present study, the effectiveness of these extracts can vary depending on the plant species, the type of extract, the target insect, and the applied dose. This highlights the importance of selecting the appropriate plant species and extract type when developing botanical insecticides.

Conclusions

This research underscored the potential of medicinal plant extracts from arid regions to act as natural insecticides against *T. castaneum*. Phytochemical analysis revealed that these plants are rich in bioactive compounds such as flavonoids, terpenoids, and tannins, which may contribute to their insecticidal and antifeedant activities. Among the extracts, *A. halimus* showed the highest antifeedant activity at 31.37%, consistent with its diverse phytochemical profile. In contrast, *L. multifida* and *Sonchus oleraceus* showed lower antifeedant indices of –3% and –0.5%, respectively, but significant insecticidal activity. *G. alypum* had minimal antifeedant activity (2%) and lower insecticidal activity. However, *L. multifida*, despite its insecticidal potential, may not be practical for use as a bioinsecticide due to its small size, which requires a large number of plants to produce a usable amount of extract. Importantly, the study found no direct correlation between antifeedant activity and insecticidal efficacy, suggesting different underlying mechanisms. Overall, these findings highlight the potential of these plant extracts as viable, ecofriendly alternatives to conventional chemical pesticides and support further research into their use in sustainable pest management.

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ORIGINAL ARTICLE

Biological control potential of *Trichoderma asperellum* against *Pseudocercospora griseola*, the etiological agent of angular leaf spot in common bean (*Phaseolus vulgaris* L.)

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Abstract

Angular leaf spot disease, which is caused by the fungus *Pseudocercospora griseola*, is among the most damaging diseases affecting common bean (*Phaseolus vulgaris* L.), impacting both yield and grain quality. Because of the environmental risks associated with fungicides and the variability in the virulence of *P. griseola* isolates, biological control emerges as a promising alternative for managing this disease. This study assessed the biological control potential of *Trichoderma asperellum* 659-7, PR11, and PR12 against *P. griseola*. Additionally, changes in some biochemical parameters were also investigated. The findings revealed that the three tested strains stopped the growth of *P. griseola* during the confrontation test, achieving 100% inhibition. Furthermore, the cell-free culture filtrates from each *T. asperellum* strain hindered the mycelial growth and spore germination of *P. griseola*, with the level of inhibition depending on both the concentration of culture filtrate and the specific strain of *T. asperellum*. The most significant reduction was noted with *T. asperellum* PR11, which decreased mycelial growth by 26.33% and spore germination by 27.14% at 25% (v/v). Moreover, treating infected bean leaves with *T. asperellum* PR11 led to a reduction in disease severity by 11.32 and 22.5% at 14 and 21 days after inoculation, respectively. An increase in chlorophyll content (287.087%), total phenols (43.116%), and flavonoids (72.010%) was also observed when infected leaves were treated with *T. asperellum* PR11. These overall results endorse the effectiveness of *Trichoderma asperellum* PR11 as a biological control agent for managing bean angular leaf spot, offering an alternative and environmentally friendly strategy.

Keywords: *Pseudocercospora griseola, Phaseolus vulgaris*, *Trichoderma asperellum*

Introduction

The common bean (*Phaseolus vulgaris* L.) is among the most significant cultivated legumes in the world (Broughton *et al.* 2003), with an estimated annual production of 27.5 million tons in 2022 (FAO 2023). This important legume consumed worldwide serves as an essential source of protein, minerals, antioxidants, and bioactive compounds (Karavidas *et al.* 2022). The Common Bean Observatory (CBO) reported an

estimated annual production of 28.5 million tons of bean in 2023 (CBO 2025), 43.9% of which come from Asia, followed by North and South America (32.2%), and Africa (21.3%) (FAO 2023). According to the Global Dry Beans Production by Country 2025, Myanmar was the first producer of this legume in 2023, followed by India, Brazil, Tanzania, and China. In Cameroon, bean production in 2023 reached 390,098 tons, harvested from an area of 303,828 hectares, resulting in an average yield of 1.28 tons per hectare (CBO 2025). This yield is significantly below the plant's potential, which is approximately 2 tons per hectare, with some varieties capable of producing up to 3.2 tons per hectare (Kebede 2021; Nchanji *et al.* 2023).

The leading causes of this poor production are low soil fertility, dryness (Papathanasiou *et al.* 2022), insect attacks (Adomako *et al.* 2022), and diseases (Eke *et al.* 2019). Within these diseases, angular leaf spot (ALS) which is caused by the phytopathogenic fungi *Pseudocercospora griseola* (Sacc.) Crous & U. Braun is considered the most destructive disease affecting bean in Latin America as well as in Africa (Sartorato 2004; Crous *et al.* 2006). This disease occurs in about 80 countries in the main bean-growing regions worldwide (Ddamulira *et al.* 2014; Nay *et al.* 2019; Bi *et al.* 2023), and it is characterized by angular dark to gray spots on the stem, leaves, and pods of the bean plant (Taboada *et al.* 2022). The disease's incidence has gone up recently, resulting in significant economic losses due to monocultures and the limited genetic diversity of commercial bean varieties (Taboada *et al.* 2022). Otherwise, when environmental conditions are favorable for the pathogen and a susceptible variety is cultivated, up to 80% of the total crop production can be lost because of the disease (Nay *et al.* 2019; Wani *et al.* 2022).

Numerous control methods, including the use of fungicides, disease-free seeds, crop rotation, host plant resistance, and cultivar mixtures, have been utilized to control ALS (Ddamulira *et al.* 2014; Ddamulira 2019). However, the emergence of different pathogen races that could overcome host resistance, coupled with the lack of suitable resistance sources often compromises genetic resistance, although it is effective and affordable (Abadio *et al.* 2012; Ddamulira *et al.* 2014; Palacıoğlu *et al.* 2021). As a result, tremendous efforts have been made worldwide to find new alternatives and reduce the negative impacts of chemicals. One promising method which has minimal environmental impact is biological control (O'Brien 2017). This approach utilizes the antagonistic mechanisms of biocontrol agents such as antibiosis, mycoparasitism and competition to disrupt the pathogen's life cycle, reduce host tissue colonization, and thereby interfere with the pathogen's ability to survive (Punja and Utkhede 2003; Busby *et al.* 2016). Among biocontrol agents, endophytic fungi such as *Trichoderma* species, known to be the most important filamentous fungi used for biological control of phytopathogens (Mohiddin *et al.* 2010; Poveda *et al.* 2020), have drawn interest (O'Brien 2017; Hyde *et al.* 2019). The biocontrol strategies of *Trichoderma* are based on stimulating several mechanisms, either directly through mycoparasitism, the secretion of active molecules, antibiosis, or indirectly through competition for nutrients and space, which promotes plant growth and enhance plant defense systems (Benítez *et al.* 2004; Ferreira and Musumeci 2021). They are considered promising candidates for biological control of plant diseases due to their diversity in producing a wide range of metabolites and their ability to inhibit the activity of up to 80% of some economically important plant pathogens (Phoka *et al.* 2020; Sánchez-Montesinos *et al.* 2020). Several investigations have demonstrated the antagonistic potential of *Trichoderma* species against pathogens such as *Fusairum solani* (Toghueo *et al.* 2016; Eke *et al.* 2020), *Uromyces appendiculatus* (Abeysinghe 2009) and *Rhizoctonia solani* (Mayo-Prieto *et al.* 2020), which cause root rot, rust and web blight in common bean, respectively. However, there are few studies demonstrating the biocontrol potential of *Trichoderma* against ALS of bean, and no study has investigated the antagonistic potential of the strains 659-7, PR11, and PR12 of *Trichoderma asperellum* against *P. griseola*.

Keeping all this in view, this study was carried out to: (1) evaluate the *in vitro* interactions between *T. asperellum* strains and *P. griseola*, (2) assess the *in vivo* efficacy of the most promising strain against ALS disease, and (3) evaluate the impact of both single and dual inoculation of bean leaves with *P. griseola* and *T. asperellum* on the chlorophyll content, total phenols and flavonoids content in bean leaves.

Materials and Methods

Source of the fungal pathogen and biological control agents

The fungal pathogen *Pseudocercospora griseola* used in this study was provided by the Antimicrobial & Biocontrol Agents Unit of the Laboratory for Phytobiochemistry and Medicinal Plants Studies of the University of Yaoundé 1. It was previously isolated from infected leaves showing the symptoms of ALS disease in a field in the Ndé division of the western region of Cameroon (5°10'28.8 "N and 10°33'58.9 "E). This isolated pathogen was the most aggressive and its identification was confirmed using molecular analysis (Gael *et al.* 2025).

Three strains of *Trichoderma asperellum*, namely *T. asperellum* 659-7, PR11 and PR12 from the core

collection of the Regional Laboratory of Biological Control and Applied Microbiology of IRAD were used as biological control agents (BCA). These strains were isolated from the top 8 cm of agricultural soils in Cameroon. Genotyping of these strains based on sequencing of translation elongation factor 1-alpha (tef1) was reported, and the sequences were deposited in GenBank. Their Genbank numbers are EF186002, EF185999 and EF186001 for *T. asperellum* PR11, PR12 and 659-7, respectively (Begoude *et al*. 2007). The antagonistic potential of these strains against various fungal pathogens, including *Phytophthora megakarya* and *Pythium myriotylum*, causative agents of black pod disease of cocoa and cocoyam root rot disease, respectively, has already been demonstrated (Tondje *et al*. 2007; Mbarga *et al*. 2012; Tchameni *et al*. 2017).

In vitro Trichoderma asperellum – Pseudocercospora griseola interactions. Direct confrontation test between *Trichoderma asperellum* strains and *Pseudocercospora griseola*

The *in vitro* antagonistic activity of *T. asperellum* 659-7, PR11 and PR12, against *P. griseola* was evaluated using the direct confrontation test on potato dextrose agar (PDA, Himedia) medium, with the Comporta (1985) procedure. Every plate was incubated for 45 days at 25±2°C. Ater measuring the pathogen's radial growth, the percentages of inhibition of mycelial growth were calculated according to formula (1) given by Mokhtar and Aid (2013).

$$I\,[\%] = (1 - \frac{T}{C}) \times 100, \qquad [1]$$

where: I [%] – percentage of inhibition, C – radial growth of *P. griseola* in the control group, and T – radial growth of *P. griseola* cultured with the antagonist.

Effect of cell-free culture filtrates of *Trichoderma asperellum* strains on the mycelium growth of *Pseudocercospora griseola*

The effect of cell-free culture filtrates of the three *T. asperellum* tested on *P. griseola* mycelial growth was evaluated using the food poisoning method (Grover and Moore 1962). Cell-free culture filtrates of *Trichoderma asperellum* 659-7, PR11, and PR12 were prepared by inoculating sterile HIMEDIA® potato dextrose broth (100 ml) with a 5 mm diameter disc of young mycelial agar plugs of each actively growing *T. asperellum* strain at 150 rpm in a rotary shaker (WiseShake®) for 10 days at 25 ± 2°C. The cultures were then filtered through Whatman filter paper No. 1 to remove the mycelium, and the culture filtrate was sterilized by passing through 0.20 µm millipore microbiological filter membranes. Melted PDA medium (at 45 ± 5°C) was amended with 12.5% and 25% (v/v) of the cell-free culture filtrate of each *T. asperellum* strain and poured into Petri plates. After cooling and solidification, a 7 mm pathogen mycelial disc was centered on the plate for 60 days in the dark. The Petri dishes with sterilized PDA without cell-free culture filtrates served as the control, and three replicates were performed for each treatment. The pathogen's colony's diameter was determined, and inhibition of mycelial growth of *P. griseola* by cell-free culture filtrates of the biocontrol agents was evaluated according to the following formula (2):

$$MGI\,[\%] = \frac{(A-B)}{A} \times 100, \qquad [2]$$

where: *MGI* – the mycelial growth inhibition, *A* – the radial growth of *P. griseola* in the control group, and *B* – to the growth of *P. griseola* in the treated group (Sreedevi *et al*. 2011).

Effect of cell-free culture filtrates of *Trichoderma asperellum* strains on the spore germination of *Pseudocercospora griseola*

To evaluate the impact of cell-free culture filtrates from *T. asperellum* 659-7, PR11 and PR12 on spore germination of *P. griseola*, the M38-A2 broth macrodilution assay described by Toghueo *et al*. (2016) was utilized. Glass tubes of 1.5 ml containing 500 µl and 625 µl of sterile potato dextrose broth (PDB, Himedia) mixed with 250 µl and 125 µl cell-free culture filtrates of *T. asperellum*, respectively. Then, a conidial suspension of *P. griseola* (250 µl) prepared at 2×10^4 spores/ml was added to each tube to complete the volume to 1 ml. Three replications were performed for each treatment, with the tubes containing PDB medium and *P. griseola* serving as the negative control. After 48 h of incubation in the dark at 25±2°C, the mixture was examined microscopically. Conidia with elongated germ tubes were considered germinated, and formula 3 below was used to calculate the percentage of inhibition of the germination of conidia (*ICG*%).

$$ICG\,[\%] = \frac{(G_c - G_t)}{G_c} \times 100, \qquad [3]$$

where: *ICG* [%] – percentage of inhibition of conidia germination, G_c – germination in control tubes and G^t – germination in treated tubes.

In vivo evaluation of the selected *Trichoderma asperellum* PR11 strain for the biological control of angular leaf spot

Seed planting and inoculum preparation

Bean seeds of susceptible cultivar GLP 190 (Gael *et al.* 2025) were surface sterilized by soaking in 3% (v/v) sodium hypochlorite (NaOCl) solution for five minutes. Afterwards, they were rinsed three times with sterilized water before being sown in plastic pots (4 cm deep) filled with 5 kg of a sand-soil mixture (1 : 3 v/v) that had been sterilized at 121°C for 1 h in an autoclave. For each treatment, three pots were used, with four bean seeds sown in each pot. The pots received daily watering until the first trifoliate leaf was entirely developed.

An adjusted version of the procedure outlined by Pastor-Corrales *et al.* (1998) was used to produce the spore suspension of *P. griseola*. Sterile distilled water was added to 14-day-old cultures of *P. griseola* that were cultivated in PDA medium, and the spores were released by scraping the medium's surface with a flame-sterilized spatula. A pair of cheesecloth layers was employed to filter the resultant suspension, and a hemocytometer was used to determine its concentration. Sterile distilled water was then added to adjust the concentration to 2×10^4 conidia per ml.

Pathogen inoculation and treatment

Seventeen days after sowing, the first trifoliate leaves of each plant were sprayed on the upper and lower sides with the spore suspension of *P. griseola* (2×10^4 conidia/ml) till running off. The plants were then placed in a highly humid chamber (relative humidity > 90%) for three days to induce sporulation and subsequently transferred to a greenhouse (Castellamos *et al.* 2016).

Before treatment with the BCA, spore suspensions of *Trichoderma asperellum* PR11 strains were prepared using a seven-day-old culture of the strain grown on PDA, and the concentration of the suspension was adjusted to 10^8 conidia/ml using sterile distilled water. Ten days after infection with *P. griseola*, when symptoms began to appear, both the top and the bottom surfaces of the bean leaves were sprayed with *Trichoderma* spore suspension. The control groups included plants treated with sterilized distilled water (uninoculated control) and those infected with the pathogen only (untreated control). For each treatment, three pots (12 plants) were used, and the experiment was conducted in a completely randomized block design. Treatments were coded as follows: Control = plants treated with sterile distilled water; Pg = plants infected with *P. griseola* only; and Pg + PR11 = plants infected with *P. griseola* and treated with *Trichoderma asperellum* PR11.

Disease rating

At 14 and 21 days following infection with *P. griseola*, disease parameters were assessed. Disease incidence represented the total number of diseased plants as a percentage of all plants in each treatment (Nutter *et al.* 1991), and disease severity was assessed visually according to the 1–9 CIAT scale described by Van Schoonhoven and Pastor-Corrales (1987) and modified by Cajiao *et al.* (2023). Here, 1 = absence of visible symptom; 3 = presence of small lesions (less than 2 mm) that cover about 2% of the leaf area; 5 = presence of more typical medium-sized, angular lesions (≥ 4 mm) that make up about 5% of the leaf area; 7 = a lot of big, sporulation-filled lesions that take up almost 10% of the leaf area. The affected leaves turn chlorotic, and lesions grow on the underside of the leaves, forming gray patches with a lot of sporulation; and 9 = coalescent lesions that cover at least 25% of the leaf area and have high, gray-colored sporulation on the leaf undersides. Defoliation from chlorotic leaflets is strong and occurs early.

Effect of inoculation of bean leaves on some biochemical parameters

Total chlorophyll content

With minor adjustments, the method outlined by Arnon (1949) was used to quantify the amount of chlorophyll. For extraction, 10 ml of ice-cold 80% acetone (v/v) was used to motor-crush 0.5 g of the first trifoliate bean leaf of each treatment. Following a 5-minute centrifugation at 10,000 rpm, the mixture was collected, and the optical density (OD) of the supernatant for chlorophyll a and b was analyzed at wavelengths of 645 and 663 nm, respectively. The chlorophyll content for each instance was determined using formulas 4 through 6 below:

$$\text{Chlorophyll a [mg/g]} = \frac{[12.7\ (\text{OD } 663) - 2.69\ (\text{OD } 645)] \times V \times W}{1000}, \quad [4]$$

$$\text{Chlorophyll b [mg/g]} = \frac{[22.9\ (\text{OD } 645) - 4.68\ (\text{OD } 663)] \times V \times W}{1000}, \quad [5]$$

$$\text{Total chlorophyll [mg/g]} = \frac{[20.2\ (\text{OD } 645) - 8.02\ (\text{OD } 663)] \times V \times W}{1000}, \quad [6]$$

where: *V* – volume of the extract [ml], *W* – weight of the fresh leaf [g] and *OD* – optical density.

Extraction of total phenolic compounds and quantification

The method modified by Mujica *et al.* (2009) was used to extract total phenols.

The Folin-Ciocalteau method developed by Singleton and Rossi (1965) was used to quantify total phenolic compounds. The absorbance was measured at a wavelength of 765 nm, and the findings were reported as micrograms of gallic acid equivalent (GAE) per gram dry weight. Gallic acid was used as a standard.

Total soluble flavonoids

The method developed by Dewanto *et al.* (2002), which uses catechin as a standard, was used for flavonoids quantification. After thoroughly mixing the solution, the absorbance was determined at a wavelength of 510 nm. The µg catechin equivalents (CE)/g of dry weight was used to express the total flavonoid content (TFC).

Statistical analysis

All statistical analyses were performed with XLSTAT 2016 software. The raw data collected underwent analysis of variance (ANOVA), and Duncan's Multiple Comparison Test was applied at 5% significance level with 95% confidence limits to assess the differences between the means.

Results

Antagonistic activity of *Trichoderma asperellum* 659-7, PR11, and PR12 against *Pseudocercospora griseola*

The results of the direct confrontation test (Table 1) showed that the pathogen (*P. griseola*) did not exhibit any growth when *Trichoderma asperellum* was present. The development of *P. griseola* was entirely suppressed by all the *T. asperellum* strains tested in Petri dishes, resulting in 100% inhibition of the mycelial growth (Fig. 1). After six days of incubation, the Petri dishes were colonized entirely by all tested *Trichoderma* strains, preventing any growth of the slow-growing phytopathogen, *P. griseola*. This was observed until 45 days after incubation. In addition, *T. asperellum* grew when the pathogen was recovered from the contact zone, indicating that *P. griseola* did not survive in the presence of *Trichoderma*.

Table 1. Dual culture between *Trichoderma asperellum* 659-7, PR11, and PR12 and *Pseudocercospora griseola* after 45 days of incubation

Trichoderma asperellum strains	Inhibition rate [%]
Trichoderma asperellum 659-7	100 ± 0.00
Trichoderma asperellum PR11	100 ± 0.00
Trichoderma asperellum PR12	100 ± 0.00

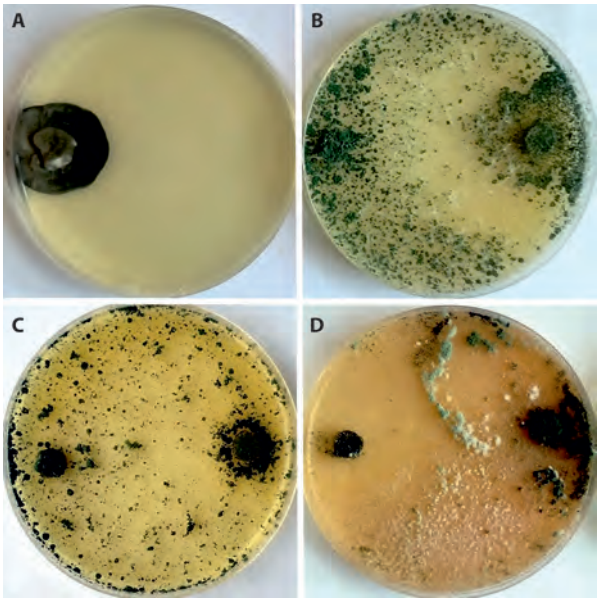

Fig. 1. Direct confrontation between *Trichoderma asperellum* PR11, PR12, 659-7 and *P. griseola* after 45 days of incubation. A – Control (*P. griseola* alone); B – dual culture between *P. griseola* and *T. asperellum* PR11; C – dual culture between *P. griseola* and *T. asperellum* PR12 and C – dual culture between *P. griseola* and *T. asperellum* 659-7

Effect of cell-free culture filtrates of *Trichoderma asperellum* 659-7, PR11 and PR12 on the mycelial growth and spore germination of *Pseudocercospora griseola*

The food poisoning technique demonstrated that the cell-free culture filtrates of all *Trichoderma asperellum* strains exhibited inhibitory effects on *P. griseola* growth (Fig. 2). The percentage of inhibition varied based on the concentration of the culture filtrate in the medium and the specific *T. asperellum* strain used. At a concentration of 12.5% of cell-free culture filtrates of *T. asperellum* 659-7, PR11and PR12, mycelial growth of *P. griseola* was inhibited by 0.8, 3.68 and 11.83%, respectively (Fig. 3). Increasing the concentration to 25% resulted in higher inhibition rates: 3.42% for 659-7, 10.01% for PR12, and 26.33% for PR11 (Fig. 3).

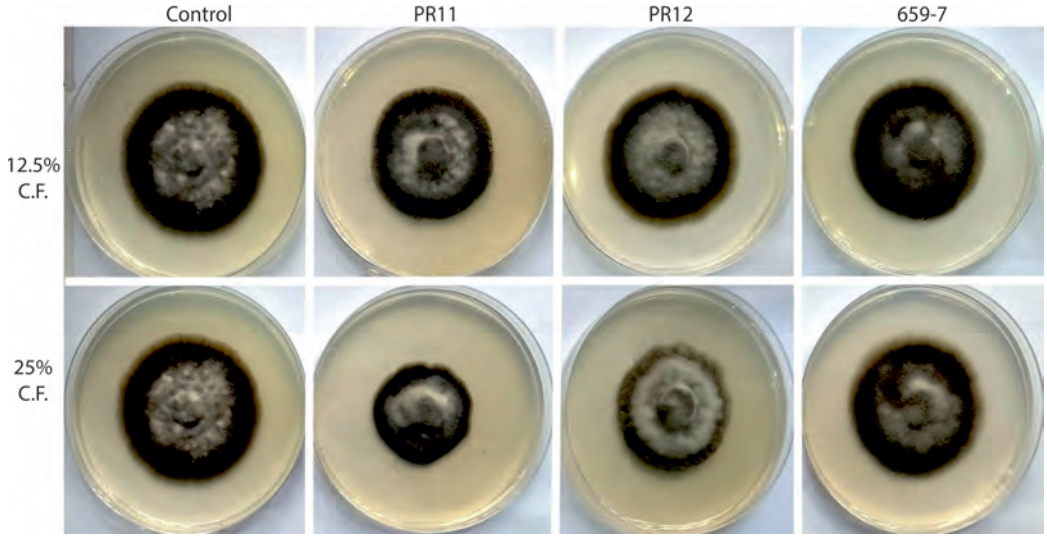

Fig. 2. Effect of different concentrations of cell-free culture filtrates (C.F) from *Trichoderma asperellum* strains (PR11, PR12 and 659-7) on the mycelial growth of *Pseudocercospora griseola*

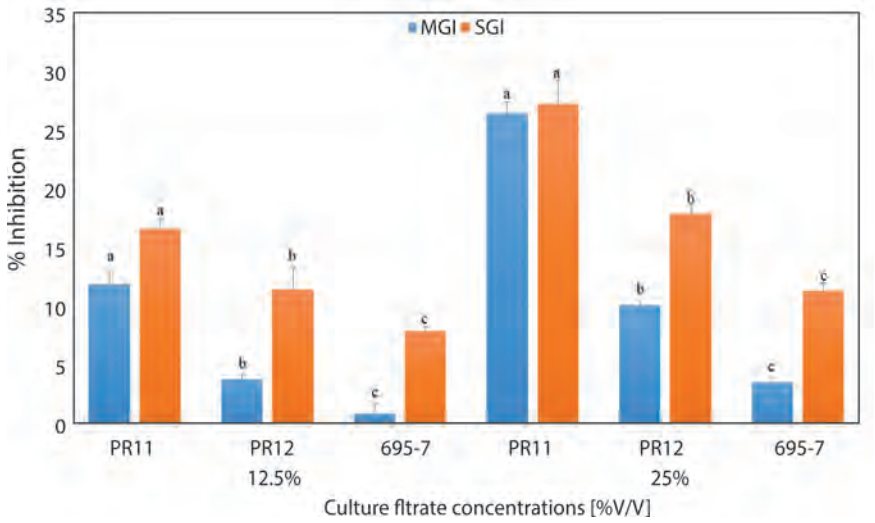

Fig. 3. Effect of different concentrations of cell-free culture filtrates (C.F) from *Trichoderma asperellum* strain PR11, PR12 and 659-7 on the mycelial growth and the spore germination of *Pseudocercospora griseola*

The process by which a fungal spore transforms from a dormant biological organism into a vegetative growing organism that can reproduce either sexually or asexually is called germination. During this process, the spore undergoes structural changes and is considered germinated once the germ tube reaches a length equal to or greater than its diameter. The effect of the cell-free culture filtrates of *Trichoderma asperellum* PR11, PR12 and 659-7 on spore germination of *P. griseola* are illustrated in Figure 3 and Figure 4. Accordingly, the percentage of inhibition of spore germination of *P. griseola* also depended on the concentration of cell-free culture filtrate and the specific strain

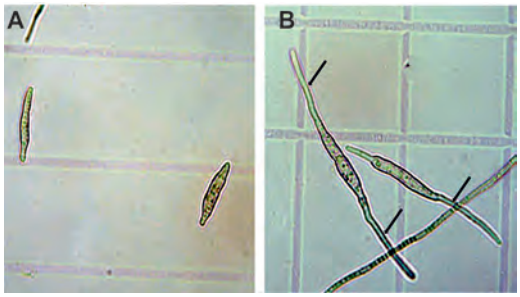

Fig. 4. Effect of cell-free culture filtrates from *Trichoderma asperellum* strain PR11, PR12 and 659-7 on the spore germination of *Pseudocercospora griseola*. Black arrows represent the germ tube. A – spore of *P. griseola*; B – germinated spore of *P. griseola*

producing this culture filtrate. At 12.5%, the highest inhibition of spore germination (16.50%) was observed with the culture filtrates of *T. asperellum* PR11, while the cell-free culture filtrates of *T. asperellum* PR12 and 659-7 inhibited spore germination by 11.37 and 7.84%, respectively (Fig. 3). When the concentration was increased to 25%, 27.14% of the *P. griseola* spores were inhibited by the culture filtrate of *T. asperellum* PR11, 17.74% by those from *T. asperellum* PR12, and 11.24% by the culture filtrate of *T. asperellum* 659-7 (Fig. 3).

In vivo evaluation of selected *Trichoderma asperellum* PR11 for the control of angular leaf spot

Bean leaves began to exhibit signs of ALS 10 days after infection by the pathogen, with symptoms becoming clearly pronounced by 14 days' post-inoculation (Fig. 5). The data in Table 2 present the effect of inoculation of bean leaves with *Pseudocercospora griseola,* both individually and alongside with *Trichoderma asperellum* PR11 on the incidence and severity of ALS 14 and 21 days after inoculation. According to Table 2, all plants infected by the pathogen developed disease during these observation periods, resulting in a 100% disease incidence, while plants treated with sterile distilled water showed no disease symptoms (0% disease incidence). However, the severity varied significantly ($p < 0.05$) depending on treatment with or without *Trichoderma asperellum* PR11. Forty days after infection with *P. griseola*, the severity of bean leaves inoculated solely with *P. griseola* was 6.36. In contrast, those treated with *T. asperellum* PR11 showed a disease severity of 5.64, indicating an 11.32% reduction in disease severity due to the application of the biocontrol agent (Table 2). On the 21st day post-inoculation, the application of *T. asperellum* PR11 resulted in a 22.5% reduction in disease severity in bean leaves inoculated with the pathogen compared to leaves infected with *P. griseola* alone. Additionally, on both the 14th and 21st day after inoculation, no symptoms were observed in the non-inoculated bean leaves, and the disease score was 1.

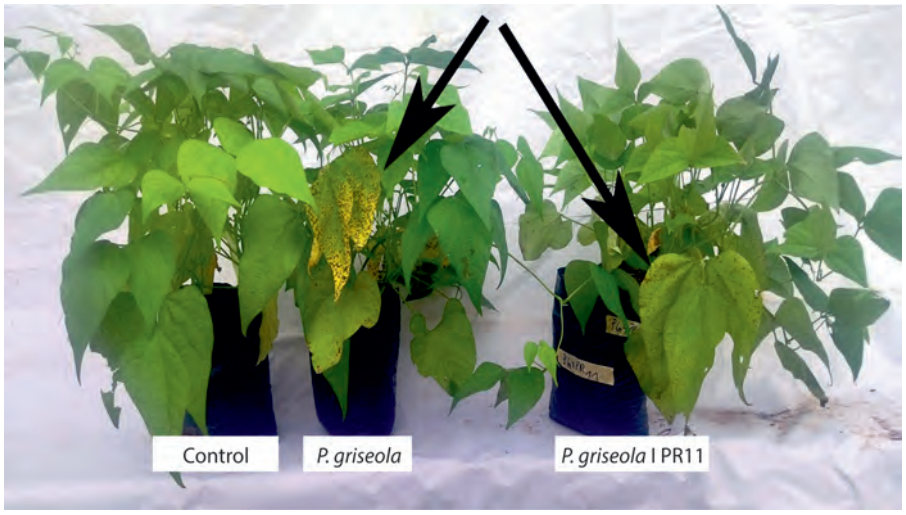

Fig. 5. Effect of single and dual inoculation of bean leaves with *Pseudocercospora griseola* and *Trichoderma asperellum* PR11 on angular leaf spot. Black arrows represent angular leaf spot lesions on bean leaves. Control: Bean leaves free from any fungal inoculation; *P. griseola*: Bean leaves infected with *P. griseola*; *P. griseola* + PR11: Bean leaves inoculated with *P. griseola* and treated with *T. asperellum* PR11

Table 2. Effect of treatment of bean leaves by *Trichoderma asperellum* PR11 on disease parameters the 14 and 21 DAI

Treatment	14 DAI			21 DAI		
	DI [%]	DS [1–9 scale]	Protection [%]	DI [%]	DS [1–9 scale]	Protection [%]
Control	0.0	1.0 ± 0.0 a	–	0.0	1.0 ± 0.0 a	–
Pg	100	6.36 ± 0.67 c	–	100	8.09 ± 0.54 c	–
Pg + PR11	100	5.64 ± 0.50 b	11.32	100	6.2 7± 0.47 b	22.5

Control: bean leaves free from any fungal inoculation; Pg: bean leaves infected by *Pseudocercospora griseola*; Pg + PR11: bean leaves inoculated with *Pseudocercospora griseola* and treated with *Trichoderma asperellum* PR11. DI = disease incidence; DS = disease severity; DAI = days after inoculation. Different letters in the same column indicate a significant difference in mean ± standard deviation values at $p \leq 0.05$

Effect of inoculation of bean leaves on some biochemical parameters

Table 3 summarizes the effect of inoculation of bean leaves with only *Pseudocercospora griseola* as well as with *Trichoderma asperellum* PR11 on the chlorophyll content, phenols and flavonoids. Compared to the control, infection of bean leaves with *P. griseola* alone resulted in an 85.238% decrease in chlorophyll, while a 42.887% reduction was noted in bean leaves infected with *P. griseola* and treated with *T. asperellum* PR11.

Table 3. Impact of single and dual inoculation of bean leaves by *Pseudocercospora griseola* and *Trichoderma asperellum* PR11 strain on the chlorophyll content, total phenols and flavonoids

Treatment	Chlorophyll [mg/g fresh weight]	Phenol [µg GAE/g dry weight]	Flavonoid [µg CE/g dry weight]
Control	0.210 ± 0.016 a	7.122 ± 1.142 c	51.241 ± 4.315 c
Pg	0.031 ± 0.008 c	18.397 ± 0.587 b	80.315 ± 3.615 b
Pg + PR11	0.120 ± 0.006 b	26.329 ± 2.198 a	138.815 ± 2.797 a

Control: Bean leaves free from any fungal inoculation; Pg: Bean leaves infected by *P. griseola*; Pg + PR11: Bean leaves inoculated with *P. griseola* and treated using the strain PR11 of *T. asperellum*. GAE – gallic acid equivalent; CE – catechin equivalents. The same superscript letter indicates that the mean ± standard deviation values of the same dependent variable do not differ significantly at $p < 0.05$

In contrast to chlorophyll content, inoculating bean leaves with the pathogen, either individually or together with the biocontrol agent, significantly increased the accumulation of phenolic compounds and flavonoids ($p < 0.05$). Untreated infected bean leaves showed a 158.42% increase in total phenolic compounds, while treated infected leaves exhibited an even greater increase of 269.80% in comparison to the control. Additionally, the flavonoid content increased by 56.73% in bean leaves infected solely with *P. griseola* and by 170.90% in those infected and treated with *T. asperellum* PR11, compared to the control.

Discussion

Angular leaf spot is a highly destructive disease that severely impacts bean quality and productivity globally (Nay *et al*. 2019). The need to develop eco-friendly and durable strategies for managing this disease led to the investigation of the biological control capability of three strains of *T. asperellum* against *P. griseola*. All the tested *T. asperellum* strains (659-7, PR11 and PR12) completely stopped *P. griseola* during the confrontation test. This result confirms the antagonistic potential of these biological control agents against plant-pathogenic fungi, aligning with research conducted by Mbarga *et al*. (2012), who showed that those strains reduced by up to 66% the mycelial growth of *Pythium myriotylum*. Wu *et al.* (2017) also characterized a novel strain of *Trichoderma asperellum* GDFS1009 and demonstrated that it reduces by 80.82% the growth of *Fusarium oxysporum* f. sp. *cucumerinum* during the antagonistic test. *Trichoderma* species are fast-growing fungi that grow rapidly and absorb the nutrients that the pathogenic fungus needs to thrive, leading to nutrient depletion and preventing the pathogenic fungus from growing and reproducing (Guo *et al*. 2019; Bazghaleh *et al.* 2020; Halifu *et al.* 2020). In addition, *Trichoderma* directly invades or wounds the mycelium of phytopathogenic fungi, resulting in changes such as growth abnormalities, shortening, rounding, protoplasmic shrinkage and breakdown of the cell membrane of the pathogen cells (Yao *et al.* 2023). These mechanisms could explain the inhibition caused by the three strains of *T. asperellum* tested.

Furthermore, the ability of *Trichoderma* to fight against plant pathogens is associated with additional mechanisms of action involving the production of antagonistic substances (El-Hasan *et al*. 2022). According to Saravanakumar *et al.* (2017), these antagonistic compounds released by *Trichoderma* spp. reduce the growth of the mycelium and inhibit the spore germination of phytopathogenic fungi. They include antibiotic substances and extracellular enzymes mainly found in their culture filtrates (Leelavathi *et al*. 2014). This could explain the results from this work, which showed that cell-free culture filtrates from *T. asperellum* 659-7, PR11, and PR12 reduced the growth of the mycelium, and inhibited the germination of spores of *P. griseola*, with percentages of inhibition varying depending on the quantity of the culture filtrate incorporated in the medium, along with the specific strain of *T. asperellum* involved. These results align with the findings of Alfiky (2019), who indicated that culture filtrates of *T. asperellum* diminish by 47.2% the mycelium development of *Rhizoctonia solani*, a fungal pathogen responsible for web blight in common bean. Raut *et al*. (2014) also observed reductions of 60 and 70.5% of the mycelium development of the same pathogen when *Trichoderma* T50 culture filtrate in the PDA medium was 10 and 25%, respectively. Wu *et al.* (2017) further demonstrated that the culture filtrate of *T. asperellum* GDFS1009 inhibits the mycelial development and the spore germination of *Fusarium* by 67.59 and 76.28%, respectively. On the other hand, data gathered by Nurbailis *et al*. (2019) indicated that culture filtrates of *Trichoderma harzianum* led to a 55.09% reduction in the conidia germination of *Colletotrichum gloeosporioides*. The noted decline in those two growth parameters of the used pathogen by the tested

T. asperellum strains might result from the antimicrobial compounds produced by *T. asperellum*. According to Druzhinina *et al*. (2018), *Trichoderma* species can generate bioactive substances that act antagonistically against phytopathogenic fungi. These bioactive compounds, which include enzymes and metabolites that break down cell walls, can successfully increase plant tolerance and decrease plant disease (Kubicek *et al*. 2019). For example, chitinase, a hydrolytic enzyme produced by *T. harzianum* inhibits both mycelial development and germination of *Fusarium graminearum* (Saravanakumar *et al*. 2017). Another enzyme found in the culture filtrates and produced by the strains that were tested is the β-1,3-glucanase (Tondje *et al*. 2007). This enzyme causes substantial destruction of the cell walls of the pathogen, thereby preventing spore germination and mycelium development (Sun *et al*. 2006). The variations in the inhibitory effect noted among the cell-free culture filtrates from the three tested *T. asperellum* strains may be because various isolates belonging to one species of *Trichoderma* produce distinct metabolites that have varying capacities to suppress the development of a pathogenic fungus (Wu *et al*. 2017).

The strain that showed the best antifungal activity during *in vitro* tests was subjected to an *in vivo* assay to assess its capacity to protect bean leaves against ALS. According to the results, the strain PR11 of *T. asperellum* decreased disease severity by 11.32 and 22.5%, respectively, at 14 and 21 days after inoculation. This outcome highlights the capacity of *T. asperellum* to fight against foliar diseases, and it aligns with the findings obtained by Jemo *et al.* (2023), who showed that soil treatment with *T. asperellum,* followed by foliar spraying of bean leaves, lowered the infection rate and the disease. The observed reduction in disease severity in treated infected bean leaves was associated with a significant increase in chlorophyll content, showcasing PR11's effectiveness in mitigating the harmful impacts of ALS. The increase in disease severity of ALS reduces the photosynthetic efficiency of the bean leaves, and therefore causes bean leaves to turn yellow due to chlorophyll shortage (Cardona *et al.* 1995; Taboada *et al*. 2022). Therefore, an increase in chlorophyll content may indicate how well *T. asperellum* PR11 protects the plant. In addition, infection of bean leaves with *P. griseola* followed by treatment with *T. asperellum* showed elevated phenolic compounds and flavonoids compared to leaves infected by the pathogen alone. This demonstrates their role in protecting the plant against the disease. In addition to mycoparasitism, antibiosis and competition, *Trichoderma* spp. can help plants fight against disease by induction of systemic defense (Asad 2022; Tyśkiewicz *et al*. 2022). During this induction, an accumulation of phenolic compounds, phenylalanine ammonia-lyase, and peroxidase activity have sometimes been observed (Ferrer *et al*. 2008; Król *et al*. 2015). This could explain the increase in total phenols, and flavonoids in infected bean leaves treated with the biocontrol agent compared to untreated infected leaves. These results align with those of Behiry *et al.* (2023), who observed an enhancement in total phenols in tomato leaves when the plant was previously inoculated with *Rhizoctonia solani* and then treated with *T. pubescens* compared to plants infected by the pathogen alone. In addition, Bala *et al*. (2024) also showed that foliar spraying of *Momordica charantia* by spore suspension of *Trichoderma* increases the accumulation of flavonoids and polyphenols by 50 and 17%, respectively. Abd El-Rahman and Mohamed (2014) found that when faba bean (*Vicia fabae* L.) leaves are infected with *Botrytis fabae* and treated with *T. harzianum*, the severity of chocolate spot disease is lowered by 41.54%, and the overall flavonoid content increases in comparison to leaves infected by *Botrytis cinerea* alone. Phenolic compounds, a particular category of secondary metabolites produced in plants, are crucial for defending the plant against both abiotic and biotic stresses. They serve as the primary defense mechanism used to fend off invasive pathogens in plants. Phenols are produced through the phenylpropanoid metabolic pathway and enhance plants' resistance by providing antimicrobial, antioxidant, and signaling functions. Certain phenols, such as phytoalexins and tannins, inhibit the growth of phytopathogens, including bacteria, fungi, and viruses, by disrupting their cell membranes or interfering with their enzymatic activities. Others, like salicylic acid, trigger systemic acquired resistance, providing resistance-enhancing to infected plants (Kumar *et al*. 2020).

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ORIGINAL ARTICLE

ERROA: Enhanced remora rider optimization algorithm-based AlexNet for rice leaf disease classification

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Abstract

Rice is a major food in India, playing an important role in the agricultural sector. However, various leaf diseases adversely affect rice production by reducing both quality and yield, leading to financial losses for farmers. Detecting these diseases at an early stage through automated methods can facilitate timely intervention and minimize crop damage. To classify diseases of rice, a novel method called ERROA-AlexNet was introduced. This model was designed to identify four categories of diseases such as bacterial leaf blight, rice leaf blast, brown spot, and tungro. The classification process utilized AlexNet, with its weights optimized using the Enhanced Remora Rider Optimization Algorithm (ERROA), a hybrid approach that integrated the Enhanced Remora Optimization Algorithm (EROA) and Rider Optimization Algorithm (ROA). Experimental results, assessed by utilizing a *k*-fold cross-validation technique, demonstrated that the proposed technique achieved an accuracy of 95.4%, a sensitivity of 94.3%, and a specificity of 98.1%. These results indicate that the ERROA-AlexNet approach outperformed conventional deep learning models such as RSW-Deep RNN, hybrid CNN-SVM and Deep CNN, as cited in the literature. This study focused on the promising features of DL in precision agriculture, providing an efficient and reliable solution for automatic detection of rice leaf diseases.

Keywords: AlexNet, classification, Enhanced Remora Optimization Algorithm (EROA), rice leaf diseases, Rider Optimization Algorithm (ROA)

Introduction

Rice is one of the most important foods in most of India, providing sustenance to millions of people (Chaudhari and Karunakaran 2024a). However, various diseases affecting rice plants can lead to substantial reductions in both crop production and value (Ramesh and Vydeki 2019). Early detection (Gong and Zhang 2023) and effective management is essential to prevent significant agricultural losses. Farmers often rely on traditional methods to identify and manage plant diseases, but manual detection is protracted and leads to errors. Recent advancements in technology have introduced automated solutions for disease detection (Prajapati *et al.* 2017), which provide accurate and efficient disease classification, allowing for timely intervention. Several rice leaf diseases (Haridasan *et al.* 2023), including bacterial leaf blight (BLB) (*Xanthomonas oryzae* pv. *oryzae*), tungro (*Rice tungro bacilliform virus* and *Rice tungro spherical virus*), leaf blast (LB) (*Magnaporthe oryzae*), brown spot (BS) (*Bipolaris oryzae*), and leaf smut (LS) (*Entyloma oryzae*) (Chaudhari and Karunakaran 2024b), can severely impact crop productivity if not identified early. DL models, particularly CNN (Abasi *et al.* 2023), have substantially enhanced the accuracy of disease classification

by automatically removing the features. Unlike traditional methods that require extensive manual preprocessing, CNN-based approaches (Yakkundimath *et al.* 2022) streamline the identification process, enhancing precision and speed.

This study introduced a novel approach called, ERROA-AlexNet (Chowdhury *et al.* 2020) for rice leaf disease classification. The method began with pre-processing, employing a bilateral filtering (Zhou *et al.* 2019) process to enhance quality of image. Image augmentation techniques, including random erasing and rotation, were applied to expand the dataset. Feature extraction incorporated CNN-based and statistical features, followed by classification using the AlexNet model (Lwin and Htwe 2023) trained with the proposed ERROA algorithm. ERROA integrates the Enhanced Remora Optimization Algorithm (EROA) (Wang *et al.* 2022) with the Rider Optimization Algorithm (ROA) (Binu and Kariyappa 2018) to optimize the classification process. By leveraging this approach, the study aimed to enhance the accuracy and efficiency of rice leaf disease detection, aiding farmers in increasing production of healthy crops and secure sustainable agricultural practices. The major contributions of the study were:

- Development of ERROA-AlexNet for Rice Leaf Disease Classification: A novel classifier, ERROA--AlexNet, was introduced for effective classification.
- Implementation of GrabCut Algorithm for Segmentation: The segmentation of diseased regions was performed using the GrabCut algorithm, improving the accuracy of disease identification.
- Optimized Training of AlexNet with ERROA: The AlexNet model was optimized using ERROA, a hybrid algorithm combining EROA and ROA, to achieve optimal weight adjustments for better classification performance.
- Introduction of the Rider Optimization Algorithm (ROA): ROA is a newly proposed technique motivated by the behavior of a category of riders moving towards a target.
- Enhancement of ROA through EROA: The Enhanced Remora Optimization Algorithm (EROA) integrated a Restart Strategy (RS) with ROA to improve optimization efficiency and enhance classification accuracy.

Identifying research gaps in the field of rice leaf disease classification encourages scholars to develop novel classification techniques. This section reviews previous studies on rice leaf disease classification and highlights their limitations.

Chaudhari and Malathi (2024a) presented the Fractional Remora Reptile Search Algorithm-based LeNet (FRRSA-LeNet) for classifying rice leaf diseases. This method integrates Fractional Calculus (FC), Remora Optimization Algorithm (ROA), and Reptile Search Algorithm (RSA). FRRSA-LeNet achieved a maximum testing accuracy of 90.3%, a TPR (True positive rate) of 88.3%, a TNR (True negative rate) of 90.4%, and a mean squared error (MSE) of 0.800. However, the primary limitation of this approach is its classification accuracy, which could be improved through parameter optimization.

Chaudhari and Malathi (2024b) proposed a Remora-CNN model for identifying and classifying four types of rice leaf diseases such as bacterial blight, leaf blast, brown spot, and tungro. The CNN model was trained using the Remora Optimization Algorithm (ROA), and classification was enhanced through segmentation via the K-means method with a Fractional Tangential-Spherical Kernel (FTSK) algorithm. Although the Remora-CNN method achieved an Accuracy of 92.5%, Sensitivity of 93.1%, and Specificity of 94.1%, it does not classify all known rice leaf diseases.

Daniya and Vigneshwari (2023) developed the RWW-based NN for rice leaf disease detection. The network training utilized Rider Optimization Algorithm (ROA) combined with Water Wave Optimization (WWO). However, the approach was not tested on diverse databases, limiting its generalizability. Future research should explore standard optimization techniques to assess their effectiveness.

Jiang *et al.* (2020) utilized an integration of DL and support vector machines (DL-SVM) to classify and predict rice plant diseases. While this approach effectively identified diseases and offered practical guidance for crop management, it did not significantly improve classification accuracy.

Upadhyay and Kumar (2022) proposed an efficient rice disease detection technique using CNN for disease classification. The integration of feature extraction and classification simplified the model, reducing processing time and complexity. However, the method did not significantly improve classification effectiveness.

Devi and Neelamegam (2019) applied techniques of image processing to automate rice leaf disease identification. Their approach involved back propagation neural networks, KNN, multiclass SVM, and Naïve Bayes classifier. Despite its effectiveness, the technique did not reduce the complexity of the detection process.

The confronted challenges of traditional methods are given below.

- Existing methods struggle to extract distinct and relevant features from diseased areas.
- Poor classifier selection and inefficient feature extraction reduce detection accuracy.
- The complexity of visual disease patterns makes identification challenging.
- Accurate diagnosis is complicated by multiple factors, including nutrient levels, pathogens, and environmental conditions.

- The effectiveness of rice leaf disease classification models is hindered by the limited availability of large-scale, diverse datasets.

Materials and Methods

This section introduces a novel model for the detection and classification of rice plant diseases. A schematic diagram of the developed system is provided in Figure 1. Initially, input images of the rice plants underwent a pre-processing phase to prepare them for further analysis. Then they were exposed to a segmentation process, where the GrabCut algorithm (Chaudhari and Malathi 2023, 2024a) was used to separate the relevant portions of the image. This step helped remove unwanted distortions, refining the image before it moves forward to the feature extraction process. After segmentation, the system moved to the feature extraction steps, where both CNN features (Zhang *et al.* 2016) and statistical features (Chaudhari and Malathi 2024a) were extracted from each segment. Once the segments were isolated, the CNN and statistical features were collected and compiled into a feature vector for classification. For the classification task, the AlexNet architecture was employed, with a novel optimization method called the Enhanced Remora-Rider Optimization Algorithm (ERROA) used to train the model. ERROA is a hybrid optimization (Goluguri *et al.* 2021) approach that combined the advantages of the traditional Enhanced Remora Optimization

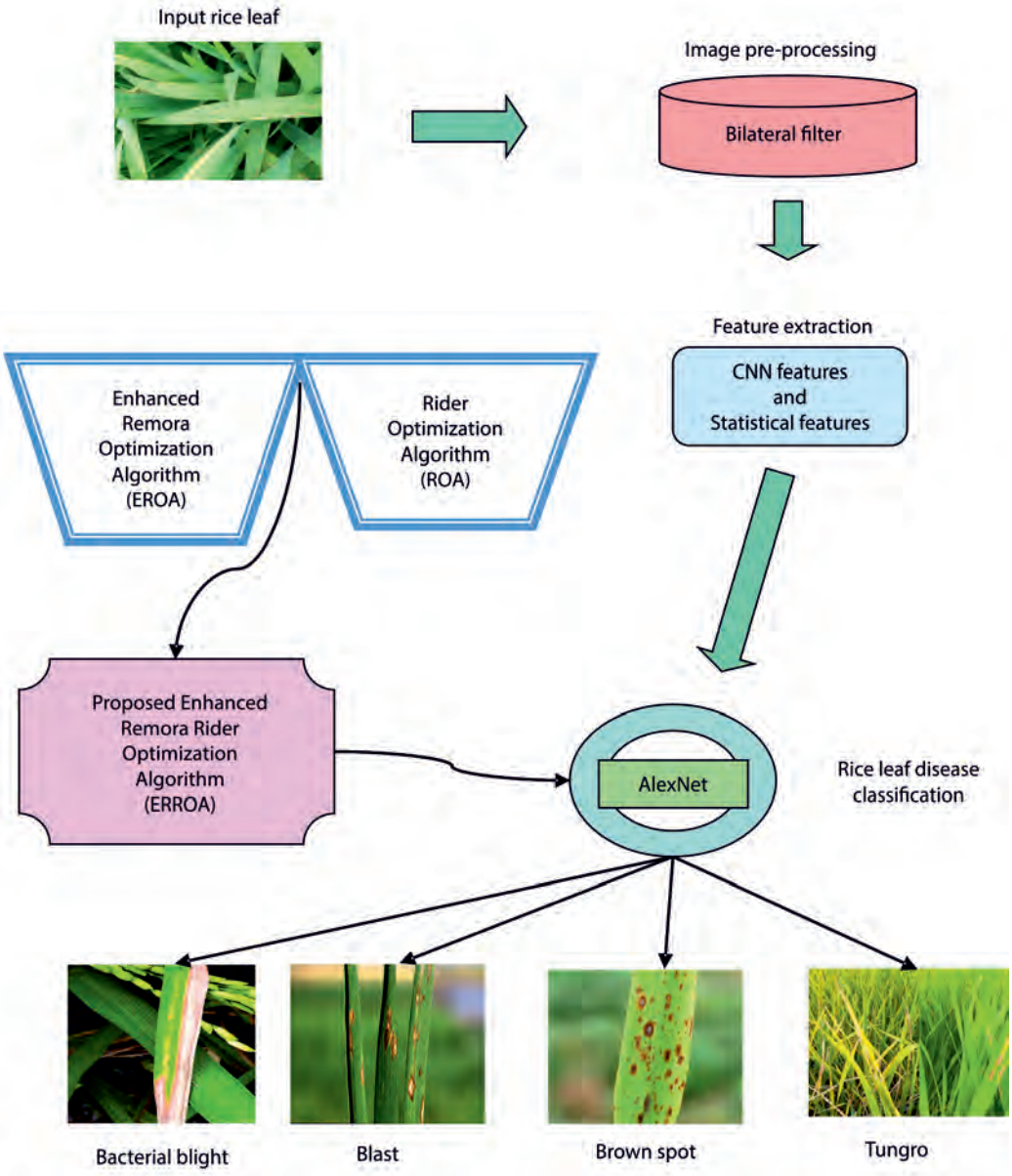

Fig. 1. Schematic view of the proposed ERROA-AlexNet for classification of rice leaf images

Algorithm (EROA) (Wang *et al.* 2022) and Rider Optimization Algorithm (ROA) (Binu and Kariyappa 2018; Daniya and Vigneshwari 2023) to effectively train the classifier and improve its performance.

Disease detection was then performed using the ERROA-AlexNet model, which benefited from the enhanced capabilities of both optimization algorithms (Mirjalili and Lewis 2016; Shadravan *et al.* 2019).

Dataset D should consist of n images, represented as:

$$D = \{K_n\};\ (1 \leq v \leq n) \quad [1]$$

where: n – all the images and K_n – the n^{th} image.

Pre-processing

In this section, pre-processing was performed using a bilateral filter (Chaudhari and Malathi 2024a; Anoop and Bipin 2019) for noise removal. Bilateral filter is a non-iterative and non-linear filter that helped to maintain edges while eliminating noise. Each pixel in the neighborhood contributed to the output based on a weighted average, where the weights were determined by both the spatial and intensity distances of the pixels.

Segmentation

Segmentation (Chen *et al.* 2021) was primarily applied to remove unnecessary portions of an image. This helped reduce computational complexity and minimized incorrect predictions. In the designed model, the GrabCut algorithm (Chaudhari and Malathi 2024a) was employed for segmentation. Following image pre-processing, segmentation was performed on an input, where the disease-affected region was extracted using the GrabCut method.

Data augmentation

Once segmentation was completed, the output underwent image augmentation (Prottasha and Reza 2022) to expand the dataset. This process helped improve model performance by increasing data diversity. In this case, rotation (Sethy *et al.* 2020) was applied to modify the image while preserving its essential features.

Feature extraction

In this study, both CNN features (Liang *et al.* 2019) and statistical features such as mean, variance, standard deviation, kurtosis, skewness, and entropy were extracted (Azim *et al.* 2021).

Thus, the extracted feature vector was represented as:

$$F_{vector} = \{F_{CNN}, F_m, F_v, F_{sd}, F_k, F_s, F_e\}, \quad [2]$$

where: F_{vector} – feature vector, F_{CNN} – CNN features, F_m – feature mean, F_v – feature variance, F_{sd} – feature standard deviation, F_k – feature kurtosis, F_s – feature skewness and F_e – feature entropy.

Proposed ERROA-AlexNet algorithm

The extracted features from the previous stages were fed into AlexNet for classification, where the training process was optimized using the proposed ERROA, a hybrid approach combining the Enhanced Remora Optimization Algorithm (EROA) and Rider Optimization Algorithm (ROA). The proposed ERROA method leveraged the strengths of both techniques, leading to improved convergence speed, and enhanced solution diversity. The structure of AlexNet, coupled with ERROA-based optimization approach, is outlined below.

Architecture of AlexNet

The selected features F_{vector} from the CNN and statistical features were used as input for the AlexNet classifier. The architecture of AlexNet contained the following different components.

Convolutional layer

This operation involved convolving a two-dimensional array of weights (filters) with an input data array. After applying each filter to the input image, feature maps were generated.

In this architecture, the ReLU activation function was employed. ReLU is a non-linear function that activated multiple neuron layers while efficiently handling back-propagation errors.

Pooling layer

The primary role of the pooling layer was to progressively reduce the spatial dimensions of feature representations. By minimizing the number of parameters and computational complexity, it helped to prevent overfitting. In this implementation, a 2 × 2 max-pooling filter with a stride of 2 was used, ensuring efficient feature extraction while preserving key spatial information.

Fully connected layer

The fully connected layer was accountable for classification and detection after the pooling layer has processed the data. To enhance performance and mitigate overfitting, a dropout layer was incorporated. This regularization technique improved generalization and ensured a more robust model. The architecture of AlexNet (Hemmer *et al.* 2018) is delineated in Figure 2.

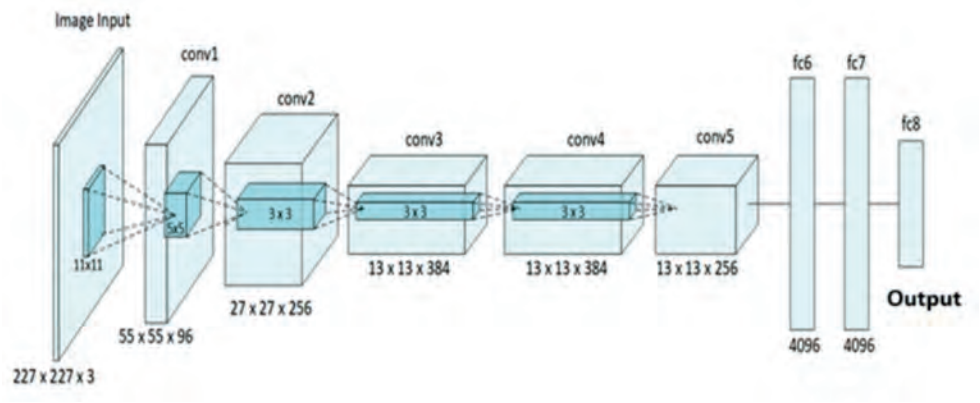

Fig. 2. Architecture of AlexNet (Hemmer *et al.* 2018)

Enhanced remora optimization algorithm

The fundamental framework of Enhanced Remora Optimization Algorithm (EROA) (Zheng *et al.* 2022) remained similar to Remora Optimization Algorithm (ROA), with the key enhancement being the addition of the restart strategy (Wang *et al.* 2022; Zhang *et al.* 2021) at the final stage of the optimization process. Restart strategies were employed in EROA to help poorly performing individuals escape local optima and prevent population stagnation.

Rider optimization algorithm

The Rider Optimization Algorithm (ROA) (Binu and Kariyappa 2018) followed the fictional computing concept to identify optimal solutions with enhanced convergence speed. The positions of the four distinct groups of riders were initialized randomly. The initialization of groups (Wang *et al.* 2019) was defined as follows:

$$M_t = \{M_t(i,k)\}; 1 \le i \le R;\ 1 \le k \le Z \quad [3]$$

where: R – the total number of riders, Z – the dimension or the number of dimensions in problem space, $\{M_t(i,k)\}$ – the location of the i^{th} rider.

To determine the final rider and ultimately the winner, the positions of all riders in the system must be updated continuously. The process of updating riders position in each group is described below. The equation governing this update is:

$$M_{t+1}^P(i,k) = \lambda[M_t(\eta,k) * \beta(k) + \\ + M_t(\vartheta,k) * [1-\beta(k)]] \quad [4]$$

where: λ, η and ϑ – random values, and β – a random variable ranging between 0 and 1 with dimensions of $1 \times Z$.

From the Remora method (Jia *et al.* 2021), the position update was as follows:

$$M_{t+1}^P(i,k) = M_t(\eta,k) + V \quad [5]$$

where:

$$V = K * (M_{t+1}^P(i,k) - W * M_t(\eta,k) \quad [6]$$

Substituting equation [6] in equation [5], we get:

$$M_{t+1}^P(i,k) = M_t(\eta,k) + \\ + K * (M_{t+1}^P(i,k) - W * M_t(\eta,k)) \quad [7]$$

$$M_{t+1}^P(i,k)(1-K) = M_t(\eta,k)(1-KW) \quad [8]$$

$$M_t\ (\eta,k) = \frac{M_{t+1}^P(i,k)(1-K)}{(1-KW)} \quad [9]$$

Substitute equation [9] in equation [4]:

$$M_{t+1}^P(i,k) = \lambda[\beta(k) * \frac{M_{t+1}^P(i,k)(1-K)}{(1-KW)} + \\ + M_t(\vartheta,k) * [1-\beta(k)]] \quad [10]$$

$$M_{t+1}^P(i,k)[1-KW-\lambda\beta(k)+\lambda\beta(k)*K)] = \\ = \lambda M_t(\vartheta,k) * [1-\beta(k)] * (1-KW) \quad [11]$$

Thus, the final equation for updating the bypass rider's position was:

$$M_{t+1}^P(i,k) = \frac{1}{[(1-KW)-\lambda\beta(k)(1-k)]} \times \\ \times \lambda M_t(\vartheta,k) * [1-\beta(k)] * (1-KW) \quad [12]$$

where: $M_{t+1}^P(i,k)$ – the new location of the bypass rider, W – the remora factor, and K – random host volume fluctuations.

Once the update process was finalized, the success rate was recalculated. All the parameters of the rider were updated accordingly in the final iteration. The process continued iteratively until the predefined

ride-off time was reached, determining the leading rider. These steps were repeated until an optimal solution was achieved.

Classification using the ERROA-based AlexNet classifier

The test input provided to the classifier should be denoted as x^t, with dimensions $1 \times w$. Utilizing the optimal weights determined through ERROA, the classification outcomes (Binu and Kariyappa 2018) were calculated as follows:

$$O^t = \varphi\left(\sum_{i=1}^{w}\left(x_i^t * D_i^O\right) + W_{iO}\right), \quad [13]$$

where: ϕ – the activation function, and D^O – the optimal weights obtained through the proposed ERROA algorithm.

The corresponding rice leaf disease classes are listed below:

$$\left.\begin{array}{ll} x^t \in \text{bacterial blight}; & \text{if } o^t = 0 \\ x^t \in \text{rice blast}; & \text{if } o^t = 1 \\ x^t \in \text{brown spot}; & \text{if } o^t = 2 \\ x^t \in \text{tungro}; & \text{if } o^t = 3 \end{array}\right\} \quad [14]$$

Results and Discussion

This section describes the results obtained using the developed ERROA-AlexNet model and evaluated using standard performance metrics. It also outlines the experimental setup, explains the dataset utilized, and highlights the effectiveness of the new method by comparing it with conventional techniques.

Experimentation setup

The developed ERROA-AlexNet model for rice leaf disease categorization was implemented using Python. Its functionality and performance were evaluated using a rice leaf disease dataset. The experiments were implemented on a system with an Intel(R), Core(TM) i7-1235U processor (2.50 GHz), 32 GB of RAM, a 512 GB SSD alongside a 1 TB HDD, 64-bit windows-11 OS (operating system). (2.50 GHz), 32 GB of RAM, a 512 GB SSD alongside a 1 TB HDD, 64-bit windows-11 OS (operating system). The experiments were implemented on a system with an Intel(R), Core(TM) i7-1235U processor (2.50 GHz), 32 GB of RAM, a 512 GB SSD alongside a 1 TB HDD, 64-bit windows-11 OS (operating system). Table 1 describes the experimental parameters used in the proposed work.

Table 1. Experimental parameters

Parameters	Value
Number of data items	5932
Batch size	16
Epochs	10
Activation function	Relu
Learning rate	0.0001
Loss	categorical_crossentropy

Description of dataset

The dataset utilized in this study was obtained from mendeley (data.mendeley.com 2024) and was comprised of a total of 5,932 images categorized into four groups of rice leaf diseases: bacterial leaf blight, leaf blast, brown spot and tungro. Each of these diseases is caused by a specific pathogen. Bacterial leaf blight is caused by *Xanthomonas oryzae* pv. *oryzae*, a bacterial species. Leaf blast is a fungal disease caused by *Magnaporthe oryzae* (also known as *Pyricularia oryzae*). Brown spot is induced by the fungus *Bipolaris oryzae*, and tungro disease results from a viral complex involving *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV), transmitted by the green leafhopper *Nephotettix virescens*. In the presented research the dataset was divided into training and testing sets using different distribution ratios: 90–10%, 80–20%, 70–30% and 60–40% for training and testing, respectively.

Experimental outcome

This section presents the experimental results obtained from the developed model. Figure 3 illustrates sample outcomes of image pre-processing, data augmentation, and segmentation techniques. Specifically, Figure 3A displays the input image, Figure 3B shows the pre-processed image, Figure 3C presents the segmented image and Figure 3D represents the rotated image.

Performance metrics

The concert of the proposed ERROA-AlexNet technique was assessing, using testing accuracy, testing sensitivity, and testing specificity.

Comparative techniques

The proposed ERROA-AlexNet technique was compared with a few existing methods, including RSW-Deep RNN (Daniya and Vigneshwari 2021), hybrid

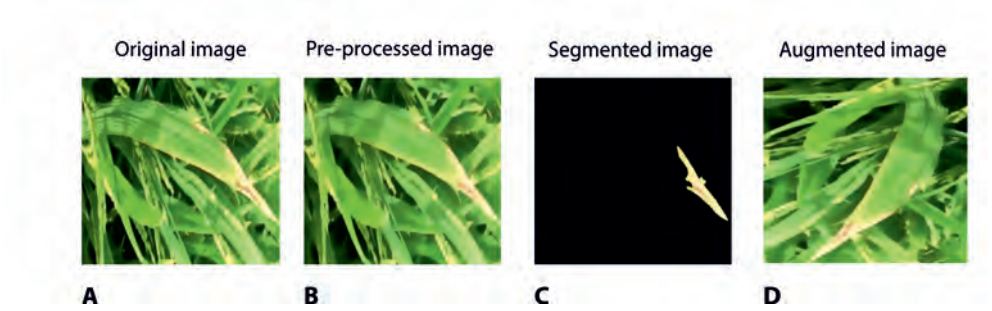

Fig. 3. Sample results: (A) input image, (B) pre-processed image, (C) segmented image, (D) rotated image

CNN-SVM (Chaudhari and Malathi 2023), and Deep CNN (Krishnamoorthy *et al.* 2021) to validate the effectiveness of the proposed approach. Specifically, the ERROA-AlexNet classifier, trained using optimization techniques, was evaluated against these models, demonstrating its superiority over conventional deep learning approaches. The selected baseline methods were chosen based on their relevance and reported effectiveness in image-based plant disease detection and classification task. Daniya and Vigneshwari (2021) introduced a Deep RNN classifier for rice plant disease detection, in which the Deep RNN is trained using a proposed RSW algorithm. Chaudhari and Malathi (2023) proposed a hybrid CNN-SVM model to detect rice leaf diseases. Krishnamoorthy *et al.* (2021) employed a pre-trained deep convolutional neural network (Deep CNN) with a transfer learning approach for detecting rice leaf diseases.

Comparative analysis

This section presents a comparative analysis, evaluating the performance of all techniques based on the percentage of training data used and the selected *k*-values.

Analysis based on training data percentage

The evaluation of the proposed ERROA-AlexNet method in terms of testing accuracy, testing sensitivity, and testing specificity, based on the percentage of training data used, is illustrated in Figure 4. Figure 4A shows the investigation of the devised scheme using testing accuracy. Examination of the devised ERROA-AlexNet scheme with testing sensitivity is described in Figure 4B, and an evaluation of the ERROA-AlexNet method with testing specificity is shown in Figure 4C. For 60% training data, the accuracy of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.670, 0.725, 0.741 and 0.785, respectively. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.665, 0.710, 0.740 and 0.775, respectively. The specificity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.681, 0.690, 0.740 and 0.765, respectively. Likewise, for 70% training data, the accuracy of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.750, 0.790, 0.812 and 0.851, respectively. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.748, 0.780, 0.810 and 0.855, respectively. The specificity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.740, 0.770, 0.814 and 0.843, respectively.

Moreover, for 80% training data, the accuracy of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.810, 0.833, 0.850 and 0.891, respectively. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.833, 0.845, 0.874 and 0.891, respectively. The specificity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.814, 0.831, 0.854 and 0.866, respectively, and lastly, for 90% training data, the accuracy of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and the proposed ERROA-AlexNet were 0.825, 0.846, 0.889 and 0.924, respectively. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.847, 0.870, 0.896 and 0.928, respectively. The specificity of RSW-Deep RNN, hybrid CNN-SVM, Deep CNN and proposed ERROA-AlexNet were 0.828, 0.841, 0.864 and 0.975, respectively.

In addition, Figure 5 illustrates the confusion matrix of the proposed ERROA-AlexNet method, evaluated using 90% of the data for training. The confusion matrix plays a crucial role in assessing the performance of classification models, particularly in multiclass classification tasks.

From the above analysis, it can be observed that the proposed ERROA-AlexNet method outperformed the RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN in terms of testing accuracy, testing sensitivity, and testing specificity across different percentage of training data.

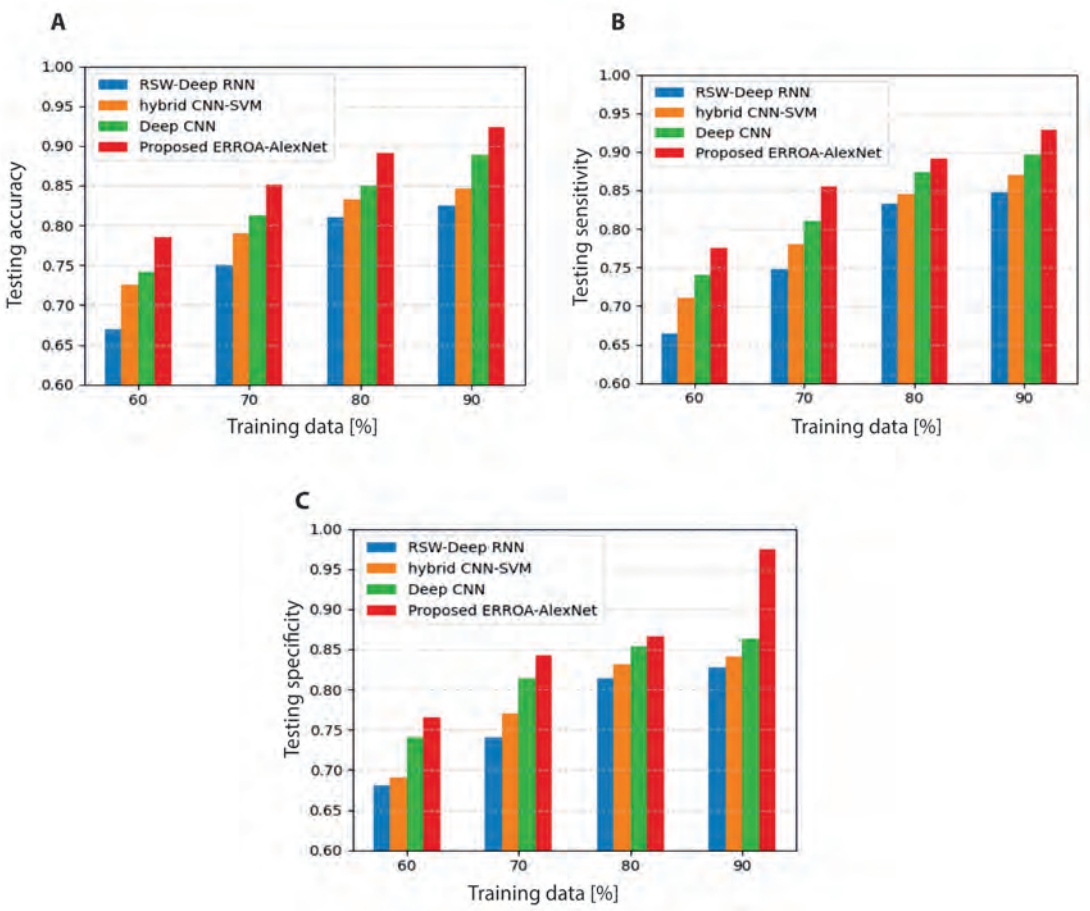

Fig. 4. Analysis based on training data percentage: (A) testing accuracy, (B) testing sensitivity, and (C) testing specificity

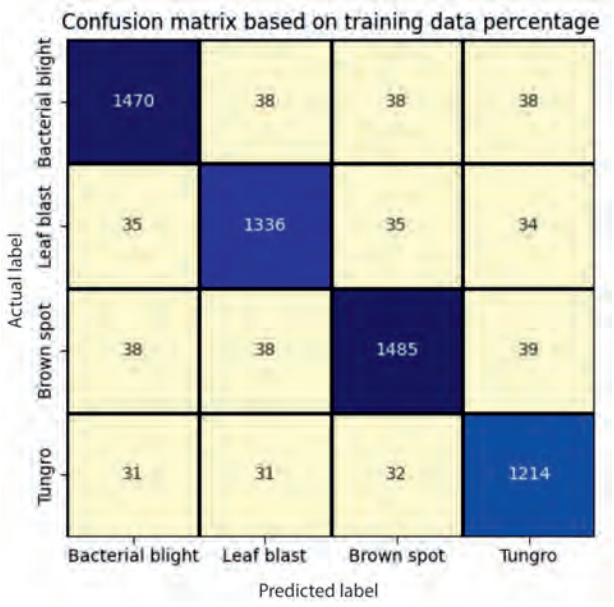

Fig. 5. Confusion matrix using 90% training data

Analysis based on *k*-fold value

Figure 6 displays the evaluation of ERROA-AlexNet method using different *k*-fold value based on testing accuracy, testing sensitivity and testing specificity metrics. Figure 6A illustrates the testing accuracy of the ERROA-AlexNet method. The examination of the devised method using testing sensitivity is given in Figure 6B. The investigation of ERROA-AlexNet using testing specificity is illustrated in Figure 6C. For *k*-fold value 4, the accuracy of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.680, 0.725 and 0.745, correspondingly, whereas the accuracy of the proposed ERROA-AlexNet was 0.772. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.721, 0.743 and 0.745, correspondingly, whereas the sensitivity of proposed ERROA-AlexNet was 0.770. The specificity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.682, 0.690 and 0.740, correspondingly, whereas the specificity of proposed ERROA-AlexNet was 0.762. Likewise, for *k*-fold value 5, the accuracy of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.745, 0.775 and 0.800, correspondingly, whereas the accuracy of the proposed ERROA-AlexNet was 0.831. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.780, 0.810 and 0.825, correspondingly, whereas the sensitivity of the proposed ERROA-AlexNet was 0.856. The specificity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.753, 0.776

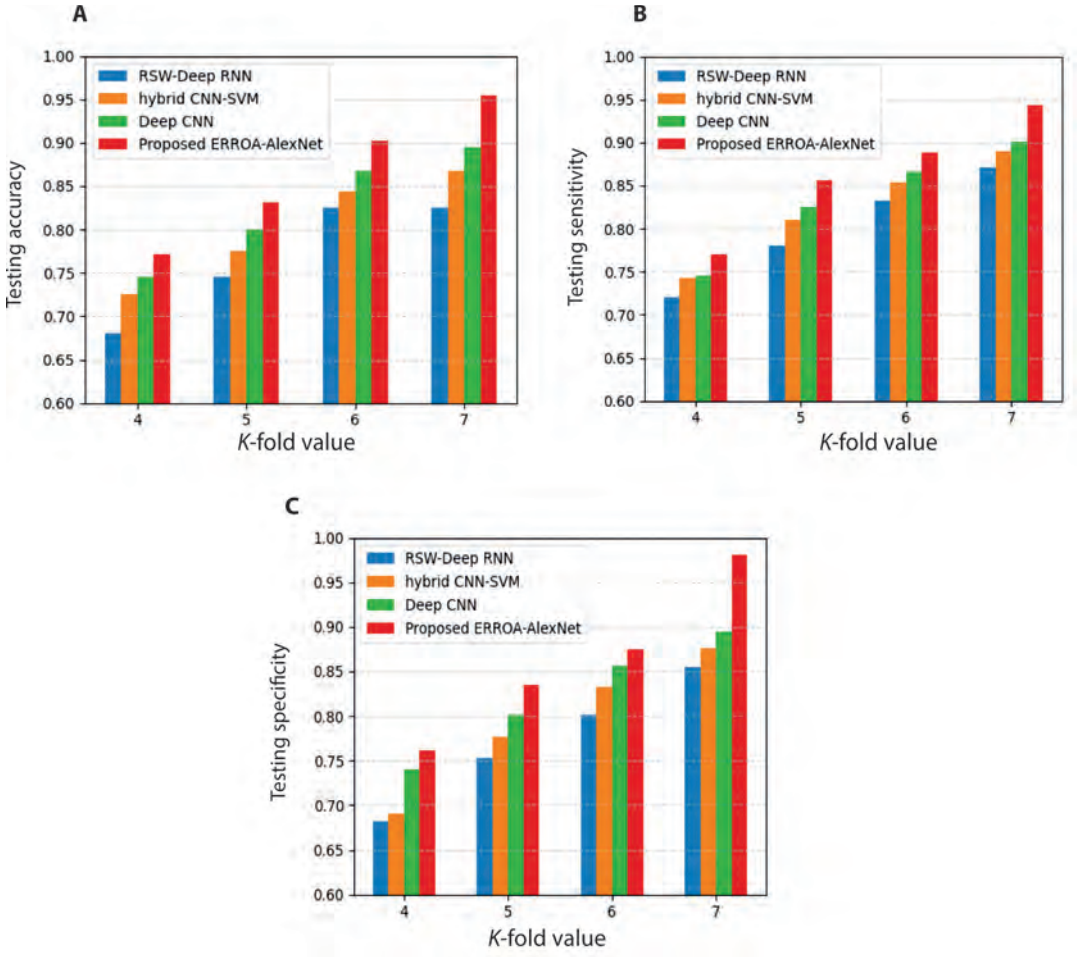

Fig. 6. Analysis based on *k*-fold value: (A) testing accuracy, (B) testing sensitivity, and (C) testing specificity

and 0.802, correspondingly, whereas the specificity of the proposed ERROA-AlexNet was 0.835.

Moreover, for *k*-fold value 6, the accuracy of RSW--Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.825, 0.844 and 0.867, correspondingly, whereas the accuracy of the proposed ERROA-AlexNet was 0.902. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.833, 0.854 and 0.866, correspondingly, whereas the sensitivity of the proposed ERROA-AlexNet was 0.889. The specificity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.802, 0.832 and 0.856, correspondingly, whereas the specificity of the proposed ERROA-AlexNet was 0.875 and lastly, for *k*-fold value 7, the accuracy of RSW--Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.825, 0.867 and 0.895, correspondingly, whereas the accuracy of the proposed ERROA-AlexNet was 0.954. The sensitivity of RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.871, 0.890 and 0.901, correspondingly, whereas the sensitivity of the proposed ERROA-AlexNet was 0.943. The specificity of RSW--Deep RNN, hybrid CNN-SVM, and Deep CNN were 0.855, 0.876 and 0.895, correspondingly, whereas the specificity of the proposed ERROA-AlexNet was 0.981.

In addition, Figure 7 illustrates the confusion matrix of the proposed ERROA-AlexNet method, evaluated with $k = 7$.

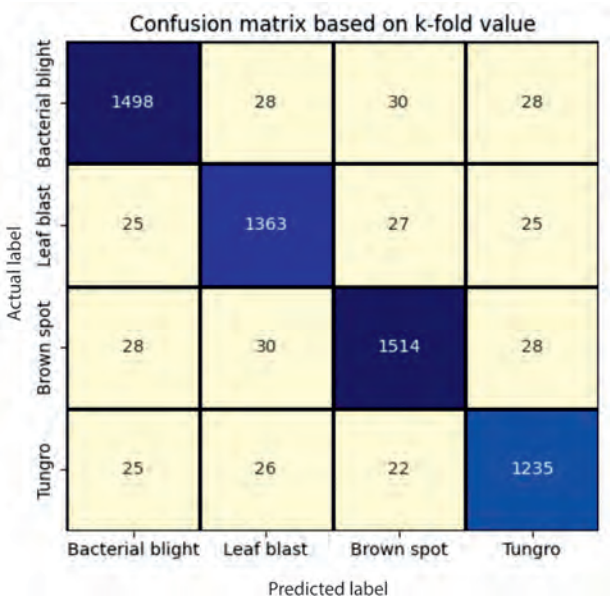

Fig. 7. Confusion matrix using *k*-fold value ($k = 7$)

Table 2. Comparative performance of proposed methods based on evaluation metrics

Analysis type	Metrics	Deep RNN	CNN-SVM	Deep CNN	Proposed ERROA AlexNet
Training data = 90%	testing accuracy	0.825	0.846	0.889	0.924
	sensitivity	0.847	0.870	0.896	0.928
	specificity	0.828	0.841	0.864	0.975
k-fold value (k = 7)	testing accuracy	0.825	0.867	0.895	0.954
	sensitivity	0.871	0.890	0.901	0.943
	specificity	0.855	0.876	0.895	0.981

Comparative discussion

Table 2 displays the comparative performance of the proposed ERROA-AlexNet method with conventional techniques with respect to evaluation metrics by modifying the percentage of training data and *k*-fold value. The table clearly revealed that the devised ERROA-AlexNet method achieved the maximum testing accuracy of 95.4%, testing sensitivity of 94.3%, and testing specificity of 98.1%, based on *k*-fold value. The experimental results indicated a significant performance improvement was achieved using the proposed model.

From the above analysis, it can be seen that the proposed ERROA-AlexNet method demonstrated superior performance compared to RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN in terms of testing accuracy, testing sensitivity, and testing specificity, based on different k-fold values.

Conclusions

In this paper, a novel ERROA-AlexNet was proposed for the detection and classification of four rice leaf diseases such as bacterial leaf blight, leaf blast, brown spot and tungro. The ERROA algorithm was invented by merging EROA and ROA optimization algorithm, which can be resorted to train the AlexNet classifier for finding the optimal weights. Once the classifier was trained on the rice leaf disease training dataset, it was evaluated for performance accuracy. After selecting optimal weights, the ERROA-AlexNet method gave classification results with testing accuracy of 0.954, testing sensitivity of 0.943, and testing specificity of 0.981. The investigational outcomes were analyzed and it was found that the ERROA-AlexNet method performed better than the traditional models such as RSW-Deep RNN, hybrid CNN-SVM, and Deep CNN.

In the future, additional features such as color, texture and shape descriptors, along with other deep learning models and larger dataset sizes, will be investigated to enhance the performance of the proposed model. Furthermore, this method can be integrated with smart technologies such as agriculture IoT and mobile devices to enable real-time monitoring and detection of diseases.

Data availability statement

The data used in this study is available in Rice Leaf -Disease Image Samples Dataset at https://data.mendeley.com/datasets/fwcj7stb8r/1.

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ORIGINAL ARTICLE

The spread of alien aphid species *Cinara curvipes* and *Cinara cedri* in Europe – the impact of climate and plant trade

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Abstract

Aphids are a strict group of insects that are particularly stimulated by current climate changes. Recent modifications in their life cycles, development, migration dates and geographical ranges are attributed to changes of local climates. *Cinara curvipes*, a species trophically related mainly to *Abies* spp., has been observed in Poland since 2015, while *Cinara cedri*, feeding on *Cedrus* sp., has been observed since 2022. Their presence is always associated with mass occurrence on host plants. The aim of this study was molecular identification of the species *C. cedri* and *C. curvipes*, collected in Poland. Potential pathways of introduction and spread of these species across Europe and the world was discussed. Based on the analysis of two genetic markers (COI and EF1-α), haplotype networks illustrating the relationships between populations from different parts of Europe and the world were presented. A contrasting pattern of low intraspecific variation in *C. curvipes*, but high in *C. cedri* was demonstrated, which may be associated with the modes of reproduction, mechanisms of dispersal of these two species, as well as introgressive hybridization between *C. cedri* and aphids belonging to *Cinara (Cupressobium)*. Genetic relationships between mitochondrial haplotypes have shown that these species have reached Poland from western and southern Europe. These species have spread from their natural range mainly through imported plant material. Human activity and climate warming have enabled them to successfully settle.

Keywords: aphid, climate change, haplotype networks, spread of species

Introduction

Aphids (Hemiptera, Aphidoidea) are insects known for their polymorphism, meaning that even within a single species there are significant morphological differences between morphs (including winged and wingless morphs, females and males) and successive generations. They are pests of most cultivated plants, including vegetables, ornamental plants, orchards and forests. Over 5,000 species are found worldwide (Foottit *et al.* 2008; Jayasinghe *et al.* 2022; Favret and Aphid Taxon Community 2026).

The genus *Cinara* includes some of the largest aphid species in terms of body size. They feed on the needles and branches of coniferous plants from the pine and cypress families (Eastop 1972). More than 370 species from the *Cinara* genus have been described in the literature, with over 150 originating from North America (Eastop 1972; Favret and Voegtlin 2004; Beğen *et al.* 2019; Favret and Aphid Taxon Community 2026). In Central Europe, including Poland, 26 species of this genus have been identified (Durak 2011). Most of them

feed on specific host plants, with the most common being *Pinus*, *Abies*, and *Picea* (Meseguer *et al.* 2015). Morphological differences within the *Cinara* genus are quite subtle. A characteristic feature is their yellow--brown or dark brown coloration and relatively large body size (up to 8 mm in length). However, despite the existing morphological differences, most species in the *Cinara* genus share common biological characteristics and may coexist on the same hosts (Watson *et al.* 1999; Meseguer *et al.* 2015) which makes identifying them and diagnosing host plant damage difficult.

The number of species inhabiting temperate climates is steadily increasing, as aphid biology is strongly affected by ongoing climate change. Changes have been observed in their life cycles, development, timing of migrations and their geographical distribution (Harrington *et al.* 2007). Such expansion has been observed, for instance, in the species *Cinara tujafilina* (Del Guercio) (Durak *et al.* 2008; Durak and Borowiak--Sobkowiak 2013). This anholocyclic species has not only broadened its distribution but has also evolved morphological, behavioral, and physiological adaptations to successfully overwinter in new, colder environments (Durak 2014; Durak *et al.* 2021). Many species of aphids are invasive and are continually expanding their distribution range (Singh and Singh 2016).

Cinara curvipes (Patch 1912) is a North American species colonizing fir (*Abies* sp.). It has been introduced into Europe and is currently considered an alien invasive species. According to data from the Global Biodiversity Information Facility (GBIF), this species is primarily distributed in the Northern Hemisphere (Fig. 1). Most of the populations of these aphids have been observed in their native regions in North America (GBIF) (*Cinara curvipes* 2023), while in Europe, *C. curvipes* has been recorded in many countries, including the United Kingdom, Germany, Serbia, Switzerland, the Czech Republic, Slovakia, Slovenia, Bulgaria, Hungary, Austria, Turkey, Poland and Norway (Wieczorek *et al.* 2025). *C. curvipes* is a species that usually massively infests firs, but can also occur on other plants like cedar, Douglas fir or western hemlock (Meseguer *et al.* 2015). Particular features of this species, such as the exceptionally high fecundity of viviparous females, the production of large numbers of winged morphs over many generations and the ability to achieve high reproduction also at lower ambient temperatures, enable it to spread rapidly to new areas and adapt to new environments (Wieczorek *et al.* 2025). Its presence is observed as early as in spring, which negatively impacts the host plants. Increased uptake of phloem sap by aphids can cause physiological disturbances in photosynthesis and respiration processes, resulting in reduced sugar production. These sugars are essential for the proper development of new vegetative and generative organs. The high number of aphids colonizing shoots, especially in early spring before the vegetation season begins, causes organ deformation and growth disorders in the following years. Weakened plants become more susceptible to fungal and bacterial infections, which may lead to further damage and even plant death (Alves *et al.* 2018; Zhan *et al.* 2020; Wang *et al.* 2023). Additionally, this species secretes large amounts of honeydew, which provides a substrate for sooty mold fungi. These fungi clog the plant's stomates, limiting photosynthesis, transpiration, and gas exchange (Zvereva *et al.* 2010). *Cinara cedri* (Mimeur, 1936; 2023) is a species that feeds on trees from the *Cedrus* genus. According to data from GBIF, *C. cedri* is widespread in Europe, especially in the Mediterranean basin including Cyprus, which is presumed to be their native range (Fig. 2). In Cyprus, the populations of *C. cedri* inhabiting *Cedrus libani subsp. brevifolia* are considered to be an endemic subspecies, *Cinara cedri subsp. brevifoliae*. Individual sightings have also been recorded in North America, Argentina and Asia (GBIF) (Michelena *et al.* 2005; Binazzi *et al.* 2017; Nozaki *et al.* 2022; *Cinara cedri* 2023). *Cinara cedri* mostly feed on the previous year's shoots, causing the needles to dry out and turn red. Young trees are particularly susceptible since dried needles fall off, leading to defoliation of shoot tips and canopies. The densest colonies have been observed mainly in city centers, parks, and gardens (Oğuzoğlu and Avci 2019a, b).

Fig. 1. Parthenogenetic females of *Cinara curvipes* (A), *Cinara cedri* (B)

Both *C. curvipes* and *C. cedri* are non-native and potentially invasive species in Central Europe, whose ranges have expanded significantly in recent years. *C. curvipes* has been observed in Poland since 2015 (Hałaj and Osiadacz 2015), while *C. cedri* has been observed since 2022 (Kanturski 2022). Because their presence is always associated with mass occurrence on host plants, their introduction could lead to disturbances in biodiversity. Their early spring appearance can result in several negative consequences, both for the plants themselves and for the entire ecosystem. Moreover, their expansion is favored by a warming climate, as it has been shown that climate plays as

Fig. 2A. Distribution map of *Cinara curvipes* based on data from GBIF (GBIF.org, 2025)
Source: https://www.gbif.org/species/2073287 (*Cinara curvipes* 2023)
Fig. 2B. Distribution map of *Cinara cedri* based on data from GBIF (GBIF.org, 2025)
Source: https://www.gbif.org/species/2073431 (*Cinara cedri* 2023)

much a role in the spread of *Cinara* as the relationship with host plants (Jousselin *et al.* 2013; Wieczorek *et al.* 2025). Investigating the genetic structure of populations may contribute to a better understanding of the dispersal processes of these species.

The aims of this study were (i) molecular identification of the species *C. curvipes* and *C. cedri*, collected in Poland and (ii) investigation of the pathways of introduction and the spread of these species across Europe and the world. Analyses were performed using fragments of sequences of two genes: mitochondrial cytochrome oxidase subunit I (COI) and nuclear elongation factor 1-alpha (EF1-α).

Materials and Methods

Samples

Two aphid species, *Cinara curvipes* and *C. cedri* were collected in Poznań, Poland. Species identification was conducted based on morphological characteristics using the Blackman, Eastop (Favret and Aphid Taxon Community 2026) key. *C. cedri* was collected from its host plant, *Cedrus deodara* 'Pendula', growing in a private garden. *C. curvipes*, on the other hand, was collected from *Abies concolor* in the Botanical Garden in Poznań. The aphids were preserved in 99.8% ethanol and stored at −20°C at the University of Rzeszów until analysis.

DNA Isolation and Amplification of COI and EF1-α genes

DNA was isolated from three individuals of each species using the NucleoSpin® Tissue kit, following the manufacturer's protocol. Each individual was sequenced separately for two genes. The obtained DNA extracts were stored at 4°C until amplification. Fragments of sequences of the mitochondrial cytochrome c oxidase subunit I gene (COI) and the nuclear elongation factor 1-alpha gene (EF1-α) were used for molecular characterization of the collected individuals. For the COI gene, two primer sets were used: LCO 5′-GGTCAACAAATCATAAAGATATTGG-3′) (Folmer *et al.* 1994) and a newly designed HCOminus (5′-AAATATGCTCGTGTATCAACATCTAT-3′). To increase specificity and avoid the sequencing of artifacts, a newly

designed internal primer, LCOplus primer (5′-CAATTATATAATGTAATTGTTACAATTCATGC-3′) was used along with HCO (5′-AAATATGCTCGTGTATCAACATCTAT-3′) (Folmer *et al.* 1994). Similarly, for amplification of the EF1-α gene, two primer sets were also used: EF3 (5′-GAACGTGAACGTGGTATCAC-3′) and EF6 (5′-TGACCAGGGTGGTTCAATAC-3′) (von Dohlen *et al.* 2002), along with external primers EF3b (5′-GTGAAATCGGCAGCACCCT-3′) and EF6b (5′-CACAGAGATTTCATCAAGAACATGAT-3′). PCR reactions were performed in a volume of 20 µl, containing 1 µl of template DNA, 1 µl of each primer (10 µM), 0.8 µl of dNTPs (5 mM), 2 µl of OptiTaq buffer (EURX), 0.2 µl of Taq polymerase (OptiTaq), and distilled water. The thermal profile included an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, primer annealing at 47–53°C for 30 seconds, and elongation at 68°C for 1 minute. Final elongation was performed at 68°C for 10 minutes. PCR products were separated on a 1.2% agarose gel in 0.5× TBE buffer. Electrophoresis was conducted at 100 V. The purified PCR products [High Pure PCR Product Purification Kit (Roche)] were sent for sequencing to Genomed (Warsaw).

Sequence analysis

The sequences were assembled into contigs and checked for potential errors using DNASTAR software (Lasergene). Neighbor-Joining trees were built, using the Kimura 2-Parameter model with 1000 bootstrap replicates separately for COI and EF1-α in MEGA 11 software (Tamura *et al.* 2021). In addition to the sequences obtained in this study (Table 1), COI and EF1-α sequences of *C. curvipes, C. cedri* and other species of the genus *Cinara* sp., from different regions of the world, were retrieved from GenBank and included in the analysis (Table 1, Table S1). Sequences from *Lachnus quercihabitans* and *Lachnus roboris* were used as outgroups in COI and EF1-α trees, respectively. Haplotype networks for *C. curvipes* and *C. cedri* were constructed (Table 1) using the Median-Joining algorithm in the PopART software (Population Analysis with Reticulate Trees) (Leigh and Bryant 2015). The number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and mean number of nucleotide differences among the haplotypes (k) in the overall sample and both populations were calculated using DnaSP 6.12 (Rozas *et al.* 2017).

Table 1. Sequences of *Cinara cedri* and *Cinara curvipes* used for haplotype network analysis

Species	Gene	GenBank acc. no	Region
Cinara cedri	COI	KU321598.1 (Binazzi *et al.* 2017)	Cyprus
Cinara cedri	COI	KU321599.1[*]	Tuscany, Italy
Cinara cedri	COI	KJ433268.1[*]	Beijing, China
Cinara cedri	COI	KM501341.1[*]	Beijing, China
Cinara cedri	COI	KM501340.1[*]	Beijing, China
Cinara cedri	COI	KU754491.1[*]	Pula, Croatia
Cinara cedri	COI	KU754492.1[*]	Pula, Croatia
Cinara cedri	COI	KF649349.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	COI	KF649387.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	COI	KF649397.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	COI	KF639314.1[*]	Montferrier Lez, France
Cinara cedri	COI	KF649509.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	COI	KF649351.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	COI	KF649350.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	COI	KF639315.1[*]	Montferrier Lez, France
Cinara cedri	COI	KF639316.1[*]	Montferrier Lez, France
Cinara cedri	COI	LC700318.1[*]	Aichi, Japan
Cinara cedri	COI	LC700313.1[*]	Aichi, Japan
Cinara cedri	COI	LC700315.1[*]	Aichi, Japan
Cinara cedri	COI	LC700314.1[*]	Aichi, Japan
Cinara cedri	COI	LC700319.1[*]	Hyogo, Japan
Cinara cedri	COI	LC700312.1[*]	Aichi, Japan
Cinara cedri	COI	LC700316.1[*]	Aichi, Japan
Cinara cedri	COI	LC700317.1 [*]	Aichi, Japan

Table 1. Sequences of *Cinara cedri* and *Cinara curvipes* used for haplotype network analysis – continued

Species	Gene	GenBank acc. no	Region
Cinara cedri	COI	KR570197.1(Hebert *et al.*2016)	British Columbia, Canada
Cinara cedri	COI	KR573470.1(Hebert *et al.*2016)	British Columbia, Canada
Cinara cedri	COI	KR567761.1(Hebert *et al.*2016)	British Columbia, Canada
Cinara cedri	COI	LT600418.1(Manzano-Marín *et al.* 2017)	Brittany, France
Cinara cedri	COI	LT600419.1(Manzano-Marín *et al.* 2017)	Aragon, Spain
Cinara cedri	COI	PV848741	Poznań, Poland
Cinara cedri	EF1-α	KF693838.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	EF1-α	KF693839.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	EF1-α	KF693840.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	EF1-α	KF693980.1(Jousselin *et al.* 2013)	Montferrier Lez, France
Cinara cedri	EF1-α	KY064501.1 (Meseguer *et al.* 2017)	Montferrier Lez, France
Cinara cedri	EF1-α	FM174683.1 (Ortiz-Rivas and Martínez-Torres 2010)	Valencia, Spain
Cinara cedri	EF1-α	KM501163.1[*]	Beijing, China
Cinara cedri	EF1-α	KM501162.1[*]	Beijing, China
Cinara cedri	EF1-α	PV843800	Poznań, Poland
Cinara curvipes	COI	KR044084.1(Gwiazdowski *et al.* 2015)	Idaho, USA
Cinara curvipes	COI	KR033906.1(Gwiazdowski *et al.* 2015)	California, USA
Cinara curvipes	COI	KR032260.1(Gwiazdowski *et al.* 2015)	Washington, Canada
Cinara curvipes	COI	KR042186.1(Gwiazdowski *et al.* 2015)	New Brunswick, Canada
Cinara curvipes	COI	KR042076.1(Gwiazdowski *et al.* 2015)	New Brunswick, Canada
Cinara curvipes	COI	KR040187.1(Gwiazdowski *et al.* 2015)	New Brunswick, Canada
Cinara curvipes	COI	JF883736.1[*]	Canada
Cinara curvipes	COI	KR036122.1(Gwiazdowski *et al.* 2015)	Manitoba, Canada
Cinara curvipes	COI	KY064233.1(Meseguer *et al.* 2017)	Montferrier Lez, France
Cinara curvipes	COI	PV848739	Poznań, Poland
Cinara curvipes	EF1-α	KY064488.1(Meseguer *et al.* 2017)	Montferrier Lez, France
Cinara curvipes	EF1-α	AY472022.1(Favret and Voegtlin 2004)	Illinois, USA
Cinara curvipes	EF1-α	PV843799	Poznań, Poland

References in brackets; [*] asterisk for unpublished data, from GenBank; grey – present study

Results

Sequence analysis

The COI gene was successfully sequenced in all *C. curvipes* samples, resulting in high-quality electropherograms. Since all the sequences obtained from different individuals were identical, one consensus sequence for the species was presented. The entire fragment was 496 bp long in this species. Also, in *C. cedri* samples, a consensus sequence was obtained for all samples (437 bp). A single consensus sequence representing the EF1-α gene for *C. curvipes* was obtained, with a length of 808 bp. The EF1-α sequence obtained for *C. cedri* consisted of 518 bp. In the Polish populations, all conspecific individuals were monomorphic for the COI gene. However, when these were analyzed together with GenBank sequences, high

Table 2. Number of haplotypes and diversity indices of *Cinara curvipes* and *Cinara cedri* in a fragment of cytochrome oxidase subunit I mitochondrial gene

Parameter	*Cinara curvipes*	*Cinara cedri*
Number of sequences used	10	29
Total number of sites	387	387
Polymorphic sites (S)	2	7
Number of Haplotypes (h)	2	4
Haplotype diversity (H_d)	0.200 (SD 0.154)	0.581 (SD 0.089)
Nucleotide diversity (π)	0.00103 (SD 0.00080)	0.00650 (SD 0.00129)
Average number of nucleotide differences (k)	0.400	2.517
Total variance of k (V(k))	0.162	1.948

genetic diversity was observed in *C. cedri* haplotypes ($\pi = 0.00650$, $k = 2.517$; Table 2). In contrast, the combined *C. curvipes* dataset was notably less divergent ($\pi = 0.00103$, and $k = 0.400$).

Species identifications and relationships between species

In both *Cinara* species, COI sequences from Poland were identical with several other haplotypes from other European populations, as well as with populations from other parts of the world (Fig. 3, Table S1).

For the COI gene both *C. curvipes* and *C. cedri* formed two distinct, strongly supported, monophyletic clades, within a clade representing other species from subgenus *Cinara* (*Cinara*) (Fig. 3A), while sequences of subgenus *Cinara* (*Cupressobium*) formed a separate, monophyletic clade. Sequences from Poland grouped together with the conspecific reference sequences obtained from GenBank. Trees built using

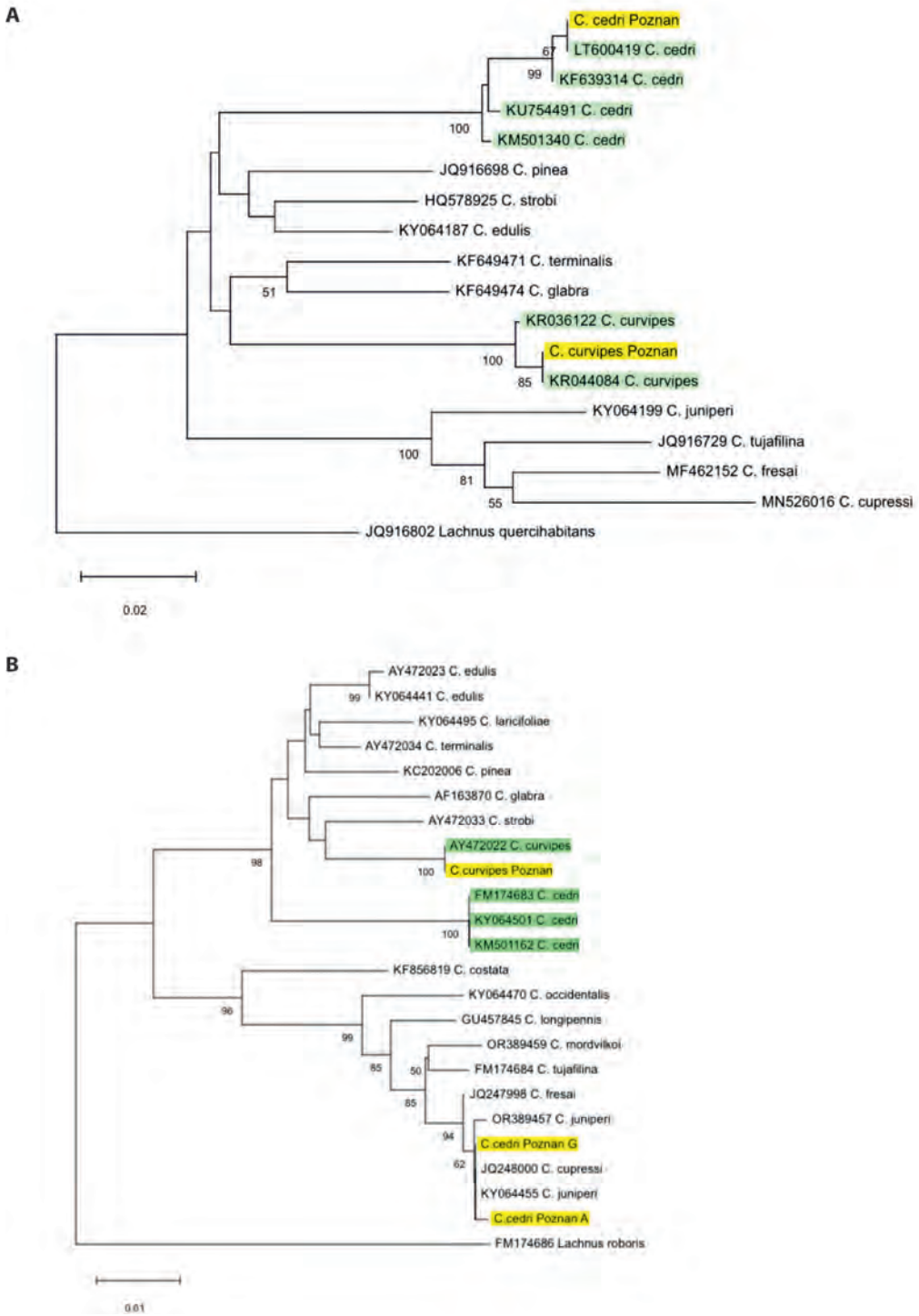

Fig. 3. Neighbor-Joining trees of *Cinara curvipes* and *Cinara cedri* for (A) mitochondrial COI gene sequences and (B) nuclear EF1-α gene sequences; bootstrap support values over 50% shown on branches

nuclear gene EF1-α grouped together the sequence of *C. curvipes* from Poland and a reference GenBank sequence of this species, while Polish sequences of *C. cedri* were grouped with sequences of *C. cupresi* and *C. juniperi* (Fig. 3B) belonging to subgenus *Cinara* (*Cupressobium*). This clade, however, also contained some sequences belonging to subgenus *Cinara* (*Cinara*) such as *C. longipennis*, *C. occidentalis* and *C. costata*.

Haplotype networks for *Cinara curvipes* and *Cinara cedri*

The haplotype networks built using two genetic markers (COI and EF1-α) illustrate the geographic patterns in the population structure of *C. curvipes* and *C. cedri*. For this analysis, all of the *C. curvipes* and *C. cedri* sequences available in the GenBank (Table 1) were used

The haplotype network of *C. curvipes* COI gene sequences (Fig. 4A) showed a low level of intraspecific variation, and two haplotypes can be distinguished: G1 and G2. The two introduced populations (Poland and France) possess a haplotype which is the most abundant in the Canadian and U.S populations (G1). The other haplotype (G2) was found only in a Canadian population from Manitoba (Fig. 4A). No variation was found in the EF1-α gene – the same haplotype was found in invasive populations from France and Poland as well as in a population from the USA. (G3) (Fig. 4B).

The haplotype network of COI sequences of *C. cedri* showed the existence of two main lineages differing by five mutations (Fig. 5A). Four haplotypes were found (H1- H4). The most abundant haplotype (H1) is present in western native populations (France and Spain) and three introduced ranges (Poland, Canada, and Japan), while haplotype H2 is present exclusively in France. The second lineage contains haplotypes from eastern native populations (Croatia, Italy, and Cyprus) but also in an introduced population in Asia (China, H3), where the derived haplotype, H4 is also present. EF1-α gene reference sequences are identical and occur both in native (France, Spain) and Asian introduced populations, while the Polish population holds solely a highly divergent haplotype H6 (Fig. 5B). The presence of the main haplotype in the populations from various countries, such as France, Spain and China, suggests that these populations share a common origin.

Fig. 4. Haplotype networks illustrating the genetic structure of populations of *Cinara curvipes* from different countries, based on the analysis of two genetic markers, COI (A) and EF1-α (B). The colors corresponding to specific countries/country regions are provided in the legend. Each circle represents a haplotype, and its size indicates the number of samples assigned to that haplotype. The lines between haplotypes represent mutations differentiating the individual haplotypes, with the number of dashes on the line imitating the number of mutations between them

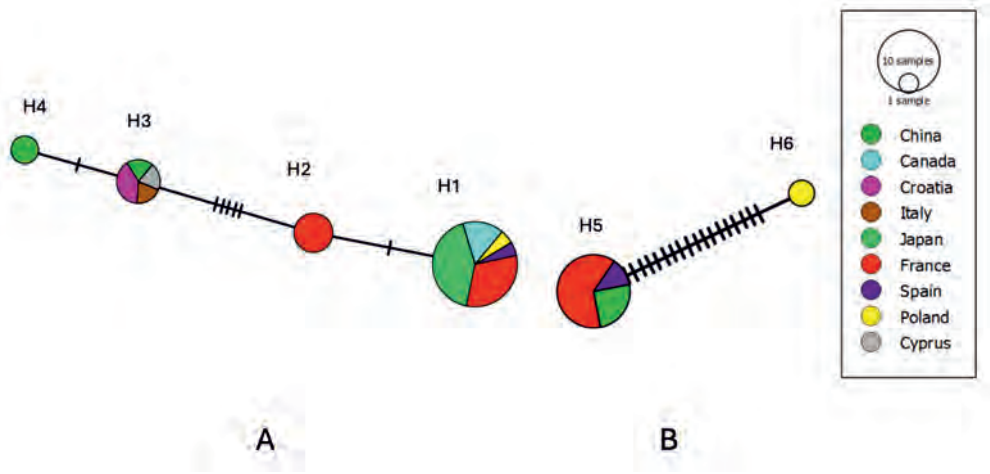

Fig. 5. Haplotype networks illustrating the genetic structure of populations of *Cinara cedri* from different countries, based on the analysis of two genetic markers, COI (A) and EF1-α (B). The colors corresponding to specific countries/country regions are provided in the legend. Each circle represents a haplotype, and its size indicates the number of samples assigned to that haplotype. The lines between haplotypes represent mutations differentiating the individual haplotypes, with the number of dashes on the line imitating the number of mutations between them

Discussion

The results from the two markers used in this study differed in power to detect the population structure of the two invasive species. The COI gene is widely used in phylogenetic studies due to both its variability, which provides a sufficient resolution at species and, in many cases, population levels, and conservativeness in certain regions, which allows designing universal primers. In insects, COI is a marker of choice in phylogenetic analyses and identification of species (Normark 2000; Hebert *et al.* 2003; Favret and Voegtlin 2004; Foottit *et al.* 2008; Chen *et al.* 2012). A significant limitation of relying solely on the COI gene is its predominantly maternal inheritance, due to its location in mitochondrial genome. It may fail to capture a complete history of taxon, especially in cases of hybridization or incomplete lineage sorting (Ballard and Whitlock 2004). The EF1-α gene, on the other hand, is located in the nuclear genome, hence, it can provide complementary information about population genetic differentiation and may better reflect the differences (Kandul *et al.* 2004; Durak *et al.* 2014).

The populations of *C. cedri* and *C. curvipes* from Poland showed genetic similarity to populations from other parts of Europe, Asia, and North America in terms of variation in the COI gene. The presented results unambiguously confirmed the presence of *C. curvipes* in Poland, placing the sequences obtained from specimens collected in Poznan in the same clades as the reference sequences of this species both in nuclear EF1-α and COI genes. On the other hand, in the individuals classified as *C. cedri* using their morphological features, while the COI gene clustered with other *C. cedri* sequences, the EF1-α sequence matched those of *C. juniperi* and *C. cupressi*. Although such incongruence between mitochondrial and nuclear sequences might suggest a hybridization event within the invasive populations, verification of this hypothesis would require analyzing additional nuclear markers and more extensive geographic sampling. The discordance between mitochondrial and nuclear genes has also been demonstrated in other aphids (Jousselin *et al.* 2024). It has been shown that phylogenetic analyses based on nuclear markers did not support the results obtained from mitochondrial analyses of many aphid subfamilies, and sometimes even failed to converge with mitochondrial phylogeny (Jousselin *et al.* 2024).

Analyses of haplotype networks revealed species specific patterns of population structure. Low genetic variability in *C. curvipes* does not allow detailed inferences about population structure of this species. Only two of the haplotypes in COI were found to have the cytochrome oxidase gene while the elongation factor gene was identical in all the individuals studied. The major haplotype, G1, is primarily associated with the Canadian and USA populations, further supporting the hypothesis of a North American source for the introduced population in Europe (Jurc *et al.* 2009). The species probably spread mainly through the migration of predominantly parthenogenetic females. Their expansion can occur through aerial plankton or with plant materials. Although the species is considered holocyclic (cyclic parthenogenesis), i.e., there is a sexual generation in its life cycle, it may also reproduce continuously parthenogenetically in areas with mild winters (Scheurer and Binazzi 2004). Previous observations of this species in Poland have not confirmed the presence of sexual generations and overwintering eggs, which could confirm the possibility that this species may also overwinter in an active form. This way of reproduction and overwintering provides *C. curvipes* with the possibility of rapid expansion. Limited genetic variation in the introduced populations might suggest parthenogenesis as the predominant mode of reproduction. Genetic bottlenecks at the front of expansion (e.g., Konopiński *et al.* 2013), along with the clonal reproduction would probably reduce variation, but limited variation in the two analyzed genes in putative source populations in North America, does not allow definite conclusions to be drawn. Populations of invasive species often show low genetic diversity between populations (Harrison and Mondor 2011). The best described species is the oleander aphid (*Aphis nerii*), which has one main haplotype that has successfully spread and dominated populations across different host plants and geographic areas (Harrison and Mondor 2011). Low genetic diversity between populations has also been observed in several major pests, including *Sitobion avenae*, *Rhopalosiphum maidis* and *Rhopalosiphum padi* (Figueroa *et al.* 2005; Guo *et al.* 2023). *R. maidis* exhibited low genetic differentiation among populations, whereas the genetic diversity of *R. padi* was overall higher than that of *R. maidis* in China as well as in Europe. The authors demonstrated that, in addition to geographical distance which can play a crucial role, the low genetic diversity between populations is also influenced by the aphid reproduction model. Obligatory parthenogenesis, which occurs mainly in *R. maidis*, reduces the chance of gene recombination, and therefore, anholocyclic populations were characterized by low genetic diversity (Guo *et al.* 2023).

C. cedri, naturally associated with the Mediterranean basin, is characterized by higher intraspecific variability. The four mitochondrial haplotypes found in this species revealed a geographical pattern. Although the two major haplogroups are separated by only two mutations, their distribution in Europe may be attributed to their independent evolutionary histories. In

the native range of the species H1/H2 haplogroup occurs in south-western Europe, while H3 was found in Italy, Croatia, and Cyprus. These two areas are known to hold independent glacial refugia for many species (e.g., Taberlet *et al.* 1998). However, to confirm this phylogeographic pattern more dense sampling and a broader range of markers would be necessary. Unfortunately, the information on EF1-α from the Balkan or Italian peninsulas leaves this part of analysis inconclusive. The sequences obtained from the Polish population of *C. cedri* suggest that the Central European population might have originated in south-western Europe. However, because the sampling of European populations is too scarce, it is not possible to disentangle the detailed expansion routes of *C. cedri*. It is uncertain if the population in western Poland emerged by gradual expansion of their range in France or if it was directly introduced by human activities. Parthenogenetic clones likely spread due to natural migration or transport via plant material (Foottit *et al.* 2008; Jurc *et al.* 2009; Nozaki *et al.* 2022). It is also possible that the populations observed in Poland resulted from expansion due to climate warming. Climate change might have allowed this thermophilic species to adapt to new areas. At the same time, the presence of suitable host plants is also important. The spread of populations is facilitated by the mobility of aphids, particularly the winged forms, which can migrate to nearby or distant locations, colonizing new areas (Dong *et al.* 1987; Xu *et al.* 2011). Previous analyses have shown close relationships between Japanese populations and those from France, Spain, and Canada, as well as genetic similarity between Chinese and Croatian populations (Ji *et al.* 2021; Nozaki *et al.* 2022). The presence of *C. cedri* in Japan was first recorded in 2021 when they were found on *Cedrus deodara* trees, which are commonly used for ornamental purposes. The appearance of this species confirms the aphid's ability to overwinter in Japan, demonstrating its adaptability and capacity to spread in the region (Nozaki *et al.* 2022).

The genetic diversity of a species can be influenced by such factors as geographical location, the host plant species and the life cycle of aphids (Caillaud and Via 2000; Vorwerk and Forneck 2006; Guo *et al.* 2023). The patterns of geographical differentiation have also been previously reported in studies in other aphid species of *Cinara*, where it was shown that geographic isolation and differences in host plants can influence genetic diversity and play a key role (Foottit *et al.* 2008; Jousselin *et al.* 2013). Human activities, such as the transport of host plants like cedars, which are increasingly planted in parks and gardens, or exotic firs and other coniferous trees planted for ornamental purposes or for Christmas tree production, may also be significant (Jurc *et al.* 2009; Nozaki *et al.* 2022). Furthermore, recent changes in the distribution of populations of both species may be promoted by climate change. Species such as *C. cedri* and *C. curvipes*, in response to rising temperatures and changing precipitation patterns, may spread in new locations outside of their natural range, appearing in areas with a moderate climate. Studies show that global warming promotes the adaptation and expansion of invasive aphid species into new areas, where environmental conditions were previously unfavorable to them (Harrington *et al.* 2007; Wieczorek *et al.* 2025). The climate changes observed in Poland, in the form of milder winters, and warmer springs and autumns, create new ecological niches suitable for *C. cedri* and *C. curvipes* aphids. A comparison of *C. cedri* and *C. curvipes* through the haplotype networks revealed significant differences in genetic structure among populations. Collectively, the results suggest that these patterns could be shaped not only by historical factors and geographic barriers, but also by ecological variables such as host plant preferences and climate change (Wang *et al.* 2017; Guo *et al.* 2023).

Conclusions

For the first time, the pathways of introduction and spread of two alien and potentially invasive aphid species, *C. curvipes* and *C. cedri* were analyzed. The genetic structure of the two species revealed lower intraspecific variation in populations of *C. curvipes*, and higher in *C. cedri*. Potential hybrid origin of the newly established population of *C. cedri* in Poland requires further investigation. It is likely that both direct human actions and climate change promote the expansion of the two species in new areas such as Central Europe.

Supplementary Materials

The following supporting information can be downloaded at Table S1: Data for aphid specimens used in this study. For each species the table includes locality, references and GenBank accession numbers for COI genetic and EF1-α markers.

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ORIGINAL ARTICLE

The spread of alien aphid species *Cinara curvipes* and *Cinara cedri* in Europe – the impact of climate and plant trade

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SUPPLEMENTARY MATERIAL

The authors are fully responsible for both the content and the formal aspects of the supplementary material. No editorial adjustments were made.

Table S1. Data for aphid species used in this study. For each species the table includes locality, references and GenBank accession numbers for COI genetic and EF1-α markers

GenBank acc. number	Gene	Species	Locality	Authors, References
LT600419	COI	*Cinara cedri*	Spain:Aragon, Mora de Rubielos	Manzano-Marin *et al.* (2016)
KF639314	COI	*Cinara cedri*	France	Coeur D' Acier *et al.* (2014)
KU754491	COI	*Cinara cedri*	Croatia: Istria	Landeka and Podnar (2016), direct submission
KM501340	COI	*Cinara cedri*	China	Chen *et al.* (2014), Direct Submission
JQ916698	COI	*Cinara pinea*	China	Chen *et al.* (2012)
HQ578925	COI	*Cinara strobi*	USA	International Barcode of Life 2010, direct submission
KY064187	COI	*Cinara edulis*	France	Meseguer *et al.* (2017)
KF649471	COI	*Cinara terminalis*	France	Jousselin *et al.* (2013)
KF649474	COI	*Cinara glabra*	France	Jousselin *et al.* (2013)
KR036122	COI	*Cinara curvipes*	Canada	Gwiazdowski *et al.* (2015)
KR044084	COI	*Cinara curvipes*	Canada	Gwiazdowski *et al.* (2015)
KY064199	COI	*Cinara juniperi*	France	Meseguer *et al.* (2017)
JQ916729	COI	*Cinara tujafilina*	China	Chen *et al.* (2012)
MF462152	COI	*Cinara fresai*	Australia: New South Wales, Bathurst	Brandley (2017), direct submission
MN526016	COI	*Cinara cupressi*	Turkey: Afyonkrahisar, Dogen	Görür and Beğen (2019)
JQ916802	COI	*Lachnus quercihabitans*	China	Chen *et al.* (2012)
AY472023	EF1α	*Cinara edulis*	USA	Favret and Voegtlin (2004)
KY064441	EF1α	*Cinara edulis*	France	Meseguer *et al.* (2017)
KY064495	EF1α	*Cinara laricifoliae*	France	Meseguer *et al.* (2017)
AY472034	EF1α	*Cinara terminalis*	USA	Favret and Voegtlin (2004)
KC202006	EF1α	*Cinara pinea*	China	Chen *et al.* (2012), direct submission
AF163870	EF1α	*Cinara glabra*	USA	Normark (2000)
AY472033	EF1α	*Cinara strobi*	USA	Favret and Voegtlin (2004)
AY472022	EF1α	*Cinara curvipes*	USA	Favret and Voegtlin (2004)
FM174683	EF1α	*Cinara cedri*	Spain	Ortiz-Rivas and Martinez-Torres (2010)
KY064501	EF1α	*Cinara cedri*	France	Meseguer *et al.* (2017)
KM501162	EF1α	*Cinara cedri*	China	Chen *et al.* (2014), direct submission
KF856819	EF1α	*Cinara costata*	China	Liu and Qiao (2013), direct submission
KY064470	EF1α	*Cinara occidentalis*	France	Meseguer *et al.* (2017)
GU457845	EF1α	*Cinara longipennis*	Republic of Korea	Kim *et al.* (2011)
OR389459	EF1α	*Cinara mordvilcoi*	Lithuania	Danilov (2023), direct submission
FM174684	EF1α	*Cinara tujafilina*	Spain	Ortiz-Rivas and Martinez-Torres (2010)
JQ247998	EF1α	*Cinara fresai*	Poland	Durak (2011), direct submission
OR389457	EF1α	*Cinara juniperi*	Lithuania	Danilov (2023), direct submission
JQ248000	EF1α	*Cinara cupressi*	Poland	Durak (2011), direct submission
KY064455	EF1α	*Cinara juniperi*	France	Meseguer *et al.* (2017)
FM174686	EF1α	*Lachnus roboris*	Spain	Ortiz-Rivas and Martinez-Torres (2010)

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ORIGINAL ARTICLE

Unraveling the fungicide resistance in *Alternaria alternata* through integrated enzymatic, phenotypic, and colony growth analysis

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Abstract

Alternaria alternata causes black spots in a variety of fruits and vegetables. There are major post-harvest losses due to a hidden fungus that develops when fruits are kept in the low temperature and appears during the fruit marketing season. This study investigated how *A. alternata* develops resistance to fungicides and how growth rates are affected by various growth media. According to the results, the resistant strain grew more slowly in the EC_{100} medium than in the control media, which showed significant differences in radial growth across media. On the other hand, the wild strain in resistant media (WS-RM) showed less development, whereas the resistant strain in wild media (RS-WM) showed more growth. Wild strains multiplied, whereas resistant strains showed decreased mycelial growth. Biochemical assays revealed significant variations between resistant and wild strains. These distinctions are highlighted by the linear correlation (R^2 = 99.38%) between protein concentrations and absorbance variation. The wild strains' control protein ratio (CPr) was 0.192, whereas the resistant strains were 0.187. The mean values of MDA (360.89 nmol · mg^{-1} protein), CAT (35.54 U · ml^{-1} protein), SOD (179.60 U · ml^{-1} protein), and tyrosinase (52.18 U · ml^{-1} protein) in resistant strains were significantly higher than those in wild strains (MDA: 179.19, CAT: 11.91, SOD: 161.36, tyrosinase: 23.90). Standard deviations for all enzymes were more significant in resistant strain, indicating increased variability. According to the pathogenicity test conducted on *Populus nigra* leaves, the resistant strain's enzymatic reactions were demonstrated by the CK leaves' continued health, the RS plants' negligible symptoms, and the WS leaves' severe necrosis. These results highlight the necessity of further investigation into the molecular pathways underpinning interactions between plants and pathogens to create focused defense strategies. Improving crop tolerance to fungus infections and environmental stressors may result in more efficient treatments, lower agricultural losses, and forest protection.

Keywords: fungicide, leaf blight fungus, *p*oplar pathogenicity, resistant strain, wild strain

Introduction

The genus *Alternaria* was first described in 1816 by Nees von Esenbeck. It belongs to the phylum *Ascomycota*, class *Dothideomycetes*, order *Pleosporales*, and family *Pleosporaceae* (Woudenberg *et al.* 2013; Waqas *et al.* 2023). Since then, 275 species of the genera *Alternaria* have been discovered, and more than 1100 identities have been published (Gou *et al.* 2022). The ideal temperature range for the growth of *Alternaria* is 22 to 30°C, whereas it may flourish in temperatures as low as 2.5 to 6.5°C, and in regions with colder temperatures as low as between 0 to –5°C (Escrivá *et al.* 2017). The fungus *Alternaria* is widely distributed and comprises pathogenic, endophytic, and saprophytic species (Gul *et al.* 2022; Li *et al.* 2023). One of the most prevalent diseases affecting a wide range of commercial crops, including crucifers (such as cabbage, cauliflower, and broccoli), beans, cotton, citrus, and tomatoes, is a leaf spot and blight disease that is brought on by the

filamentous fungus *A. alternata* (Esfahani 2019; Golian *et al*. 2023; Ahmad *et al*. 2024).

Alternaria alternata is a fungal pathogen that causes rots, blights, and leaf spots on different plant sections. It affects about 380 plant host species. Severe yield losses may result from this infection (Budziszewska and Bereś 2024; Mmbaga *et al*. 2011). For instance, early blight damage to tomato plants has been recorded to cause output losses of up to 79% in Canada. This damage can lead to crop losses of up to 30% and postharvest losses of up to 10%. Disease management strategies including cleaning and crop rotation with non-host crops, are not completely effective because of the fungus's wide host range within the *Solanaceae* family, its long-life period in plant waste, and predominant airborne transmission (Tozlu *et al*. 2018).

The fast-growing, high-yielding tree genus Poplar (*Populus* spp) is essential for the ecological and economic health of the planet. In China, the widespread *A. alternate*-induced poplar leaf blight disease has had significant adverse economic effects (Wang *et al*. 2015; Bagherabadi and Zafari 2022). Poplar, an important timber forest species in China, faces numerous pests and diseases due to its single-stranded structure. Common diseases include leaf spots, ulcers, leaf rust, leaf blight, and branch and stem rot (Duong *et al*. 2024; Yang *et al*. 2024). In the field of agriculture, during the pre- and post-harvest phases, *Alternaria* species cause considerable losses. They can even survive under low temperature storage conditions. Thus, various physiological factors, such as pH, substrate, temperature, water activity, and so on, affect mycelial growth, sporulation, and virulence either directly or indirectly (Łukaszewicz *et al*. 2021; Ahmad *et al*. 2024).

Phytopathogenic fungi have developed a variety of reactive oxygen species (ROS) producing and scavenging mechanisms for maintaining the equilibrium of the oxidative status. Fungi have developed a complex antioxidant defense system consisting of various enzyme components like peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), which are essential for surviving in various stressful environments. Reactive oxygen species (ROS) are efficiently reduced, buffered, and scavenged by these enzymes, which guarantees their survival in a variety of situations (Zhang *et al*. 2020; Gul *et al*. 2022). Cellular components and unsaturated membrane lipids are protected from free radicals by the antioxidant defense mechanism (El--Beltagi and Mohamed 2013). Another crucial element of plant defense mechanisms is malondialdehyde (MDA), particularly when the plants are reacting to various environmental stimuli. Chemically speaking, MDA is a small, reactive chemical molecule that is present in many eukaryotic organisms (Morales and Munné-Bosch 2019; Muñoz-Pérez *et al*. 2024). SOD, an important enzyme for aerobic cells, transforms harmful superoxide radicals into oxygen and hydrogen peroxide. Hydrogen peroxide is further processed by potentially dangerous enzymes like peroxidase and catalase. SOD is the first defense against reactive oxygen species (Alam *et al*. 2021).

The radial development of *A. alternata* isolates on potato dextrose agar (PDA) plates was significantly inhibited by fungicides. The systemic triazole fungicide propiconazole is used to manage a variety of fungal diseases in a range of crops, such as cereals, fruits, vegetables, and ornamental plants. With increasing fungicide doses, mycelial growth inhibition increased significantly, with the higher fungicide dose resulting in total inhibition of the examined fungus (El-Ghany 2019; Nira *et al*. 2022). "Switch" is a noteworthy fungicide combination that can be used in a blueberry management program to stop *Alternaria* rot. This fungicide has two active ingredients: fludioxonil and cyprodinil. Whereas fludioxonil belongs to the phenylpyrrole fungicide class, cyprodinil is a member of the anilinopyrimidines fungicide class (Wang *et al*. 2022; Ziedan 2022). Fludioxonil was obtained from *Pseudomonas*; fludioxonil is a chemical derivative of pyrrolnitrin, a naturally occurring substance (Oiki *et al*. 2022). Fludioxonil is classified as moderately harmful by the World Health Organization (WHO) but toxic to aquatic species. According to European classification, it has long-term negative consequences (Elskus 2012; Haegerbaeumer *et al*. 2019).

In the present investigation, *A. alternata* stress responses to environmental stressors and the mechanisms underlying their antifungal resistance were examined. Microscopy and growth media tests were among the methods used to examine the phenotypic characteristics, growth patterns, pathogenicity on poplar leaves (*Populus nigra*), and biochemical reactions of fungal strains. Antioxidative defense mechanisms, such as soluble protein levels, catalase (CAT), malondialdehyde (MDA), and superoxide dismutase (SOD), were the subject of biochemical tests. The research showed how environmental factors and fungal growth dynamics are related, highlighting the need for specialized disease management strategies and more investigation into molecular pathways to improve food security and sustainable farming.

Materials and Methods

Alternaria alternata preparation of conidia suspension

The PDA medium was prepared and preserved *A. alternate* strains (AaNEFU1) were activated. These strains had been isolated from infected poplar leaves at

Northeast Forestry University, provided by the Laboratory of Forest Pathology (Harbin, China). This strain can be found in GenBank with accession number PQ605768, although it is not a part of any official microbiological collection. After this, they were incubated at 25°C for about 15 days to allow for sporulation. After sporulation, 1 mL of sterile water was added to the *A. alternata* culture on the PDA plate. A sterile coating rod was used to gently scrape the mycelium and collect the spores. The spore suspension was transferred to a centrifuge tube and centrifuged to remove the supernatant, leaving behind the concentrated spore suspension for storage. Ten µl of spore suspension was evenly distributed on a PDA culture medium and incubated for 1 day or several days. At least 10 single colonies were cultured.

Comparative analysis of *Alternaria alternata* growth on fungicide-treated media

The objective of the present experiment was to observe the responses of fungus *Alternaria alternata* to various growth media types and environmental factors. For this experiment, four different media types were used. The CK media – PDA was used as the control and the wild strain of *A. alternata* was allowed to grow without any further treatments. Since the media EC_{100} had resistance, a fungicide was applied to the PDA. On the treated media, subsequently a strain of *A. alternata* (AaNEFU1) was inoculated that had previously been identified as resistant to this fungicide (Fludioxonil). For resistance strains, wild media (RS-WM) were used. As in the control media, PDA was used. To create an environment where the resistant strain could grow under usual conditions, it was inoculated with the same fungicide-resistant strain. The wild strain of *A. alternata* was isolated and subsequently inoculated on a wild strain's resistance media (WS-RM) medium that had been treated with the same fungicide as the EC_{100} media. Using this media setup and strain inoculation, there were three replicates of each culture medium. Every plate was incubated for 5 days. Throughout the incubation period, the colony diameter of each strain was measured regularly to monitor its growth. Each measurement was entered into a spreadsheet for further analysis after compiling all the data.

Phenotype analysis of resistant strains

Spore suspensions were examined from a resistant strain of *A. alternata* treated with an EC100 dosage of fludioxonil under a microscope. The spore germination rates were observed and recorded for both the wild and resistant strains. A PDA medium was used that contained potatoes, dextrose, and agar. For 7 days, these cultures were incubated at 25°C in an incubator. Using a fluorescent microscope, conidia size, color, septation, branching of the conidiophore, diameters of the catenulate conidia, spore germination rate, and structure were examined to characterize *Alternaria* spp. The resistant strain and the wild strain were compared in order to understand the differences between them (Saleem and El-Shahir 2022).

Determination of soluble protein content

The Coomassie brilliant blue G-250 protein staining method was used to determine the soluble protein content. A mycelium sample (0.1 g) was weighed, 1 ml of 0.9% physiological saline was added to an ice bath, ground into a homogenate, and centrifuged at 8000 × g at 4°C for 10 minutes. 150 µL of the supernatant was transferred to an enzyme-linked immunosorbent assay (ELISA) plate. Then, 200 µL of 1× G250 staining solution was added to each well of the plate (Beijing Solar Bio Science & Technology Co., Ltd., Beijing, China). The staining solution was allowed to stand at room temperature for 5 minutes, and the absorbance value at A595 nm was measured using a microplate reader. Protein concentration was calculated from a standard curve prepared under the same conditions, and three biological replicates were performed for each treatment.

Determination of malondialdehyde (MDA) content

The content of malondialdehyde (MDA) in *A. alternata* was determined using a malondialdehyde kit provided by Beijing Solar Bio Science & Technology Co., Ltd. (Beijing China), in accordance with the manufacturer's instructions.

Determination of catalase (CAT) activity

Catalase (CAT) activity was measured using a catalase kit provided by Beijing Solar Bio Science & Technology Co., Ltd. (Beijing China), in accordance with the manufacturer's instructions.

Determination of superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) activity was measured using a SOD assay kit provided by Beijing Solar Bio Science & Technology Co., Ltd. (Beijing China), following the manufacturer's instructions.

Determination of tyrosinase activity

The tyrosinase activity of *Alternaria alternata* was measured using a kit provided by Beijing Solar Bio

Science & Technology Co., Ltd. (Beijing China), following the manufacturer's instructions.

Alternaria alternata pathogenicity on poplar leaves under stress from fludioxonil

To activate the strain of *A. alternata*, a PDA plate was inoculated with the purified strain that had been kept on a PDA slant. Before using, the plate was kept at 25°C for 5 days. Young, healthy poplar leaves were collected. The leaves were rinsed with fresh water, then with 70% ethanol their surfaces were wiped gently to disinfect them. Then, the leaves were cleaned with sterile water and reserved for later use. The inoculation needles were sterilized and the poplar leaves were carefully punctured to make tiny punctures. To retain humidity, the injured leaves were placed in a sterile container lined with moist, sterile non-woven cloth and kept ready for inoculation. Fungal discs were extracted from the prepared plates of the resistant *A. alternata* strain and the wild-type *A. alternata* strain using a sterile hole punch (5 mm in diameter). The control group was consisted of resistant *A. alternata* strain + wild-type *A. alternata* strain + uninoculated, healthy leaves. The fungal discs were introduced onto the wounds made on the poplar leaves. For 5 days, the inoculated leaves were kept in an incubator set at 25°C with adequate moisture. To prevent further fungal growth and disease progression, the fungal discs were removed from all treatment groups (control, wild-type, and resistant strains) on Day 5. Following this removal, photos were taken, and measurements and documentation of the disease lesions' sizes were made. The lesions shown on Day 5 indicated the maximum disease severity the pathogen had produced during the incubation period up to that point because the removal of the fungal inoculum stopped additional fungal infection. The size of the disease lesions that formed on the leaves during incubation was measured and photographs were taken for documentation (Verma *et al.* 2020; Dreischhoff *et al.* 2023; Roy *et al.* 2023).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to evaluate the differences in growth rates between different strains and types of medium. With a significance level set at $p < 0.05$, the Tukey test for comparisons was used to establish statistical significance. Each enzyme activity measurement performed in three biological replicates for each treatment. Data were analyzed using Microsoft Excel and Origin Pro, and the results are expressed as means ± standard deviations (SD). The OriginPro Software was used to create the graphic representations (Kumar *et al.* 2019).

Results

Effects of different fungicide-treated media on *Alternaria alternata* growth rate

The results demonstrated that *A. alternata* growth rates were influenced by several media types. Figure 1 illustrates how various media types support the growth of the fungal strain. The mean radial growth of *A. alternata* varied considerably across all studied media. With a mean of 5.97 (SD = 0.07), the fungus exhibited the highest level of radial growth on potato dextrose agar (PDA) among the various treatments (media) examined. This observation is consistent with expectations because PDA is a standard medium that has no internal resistance to *A. alternata*. Conversely, media containing compounds that were resistant to *A. alternata* showed significantly reduced growth

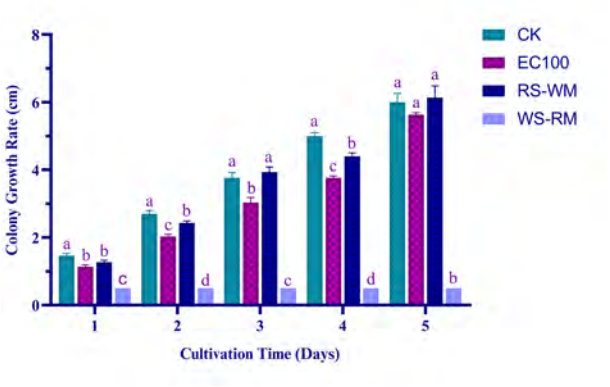

Fig. 1. Growth rate of *Alternaria alternata* on different media types over 5 days. CK – control, EC_{100} – resistant media, RS-WM – resistant strain wild media, and WS-RM – wild strain resistant media. Statistically significant differences are shown by different letters above the bars based on the Tukey test at $p < 0.05$. Bars with different letters are significantly different from ones with the same letter, which are not. The error bars represent the standard deviations (SD)

patterns. The resistant strain wild media (RS-WM) and the control media (CK) exhibited the highest radial growth rates when compared to other treated media; RS-WM's mean was 6.16 (SD = 0.14), while CK's was 5.0 (SD = 0.06). They demonstrate that under such circumstances, the growth conditions for fungal growth. With a mean of 5.63 (SD = 0.07), the EC_{100} medium supported less radial growth as well as the CK media, most likely due to the presence of resistance components. Additionally, RS-WM media grew higher than EC_{100} media, indicating that this strain may benefit from its resistant traits. In contrast to other tested media, the wild strain resistant media (WS-RM) showed restricted growth, with a mean of 0.5 (SD = 0), suggesting that the

inoculation of the wild strain on resistant media may have had inhibitory effects. In conclusion, the growth rates in CK media were almost 33%, in EC_{100} media they were 29.06%, in RS-WM media they were 33.43%, and in WS-RM media they were just 4%.

Moreover, ANOVA analysis showed that the calculated *F* value was greater than the critical *F* value (as shown in Table 1). The significant differences in the radial growth rates of *A. alternata* on different media are further illustrated in Fig. 2. Therefore, the null hypothesis was rejected and the alternative hypothesis was accepted. In ANOVA, the null hypothesis (H_0) assumes that there are no significant differences among the group means. However, if the calculated *F* value exceeds the critical *F* value, the null hypothesis is rejected. As for the alternative hypothesis (H_1), it

Table 1. ANOVA results indicating significant differences among group means ($F = 8.625594 > F\ crit = 2.539689$, $p = 1.77E-05$)

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	85.22767	4	21.30692	8.625594	1.77E-05	2.539689
Within Groups	135.8608	55	2.470197			
Total	221.0885	59				

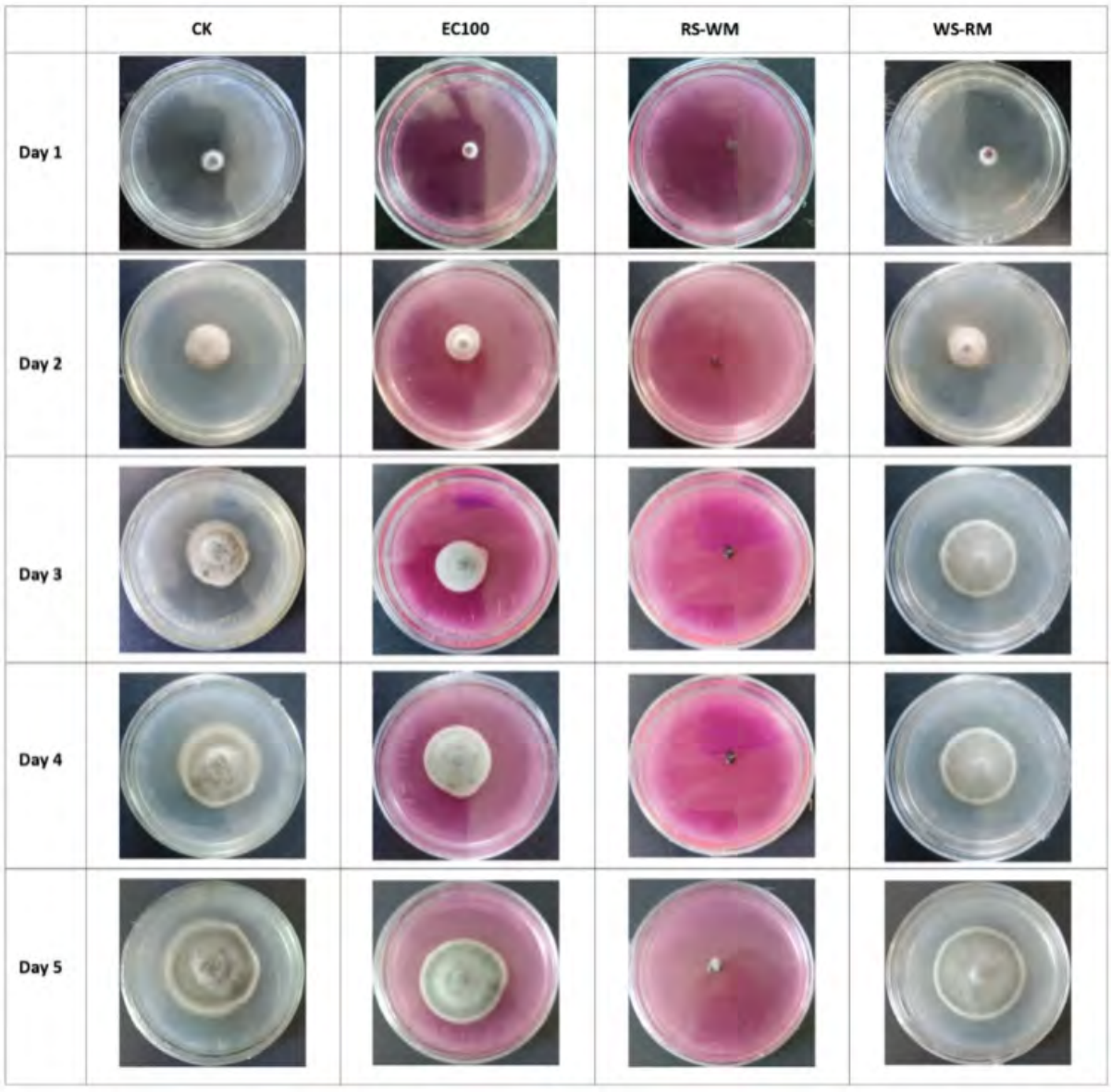

Fig. 2. Variation in *Alternaria alternata* growth pattern in different media types. Control (CK), resistant media (EC_{100}), resistant strain wild media (RS-WM), and wild strain resistant media (WS-RM)

was accepted that there was a significant difference in the group means. This result indicate that the various media types and their respective growth rates differ significantly from one another. The statistical analysis essentially highlights the variation seen in the fungal strain's ability to grow in the various growing conditions supplied by the various types of media that were used in the experiment.

Phenotype analysis of resistant and wild strains of *Alternaria alternata*

Beginning microscopic observations at a relatively small magnification – typically less than 20x – is a standard procedure to help locate the object of interest and obtain a general overview of the object. A higher power magnification is used after the object has been located and placed usually more than 60x, to enable a more thorough inspection and detailed examination of fine structural features or defining features. Investigating the phenotypic traits of *A. alternata* strains, notable differences were observed between the wild type and the resistant EC_{100} strain exposed to fludioxonil (as shown in Fig. 3). The EC_{100} strain displayed reduced mycelial growth, collapse of vertical mycelium, and uneven pigmentation due to the fungicide's interference with osmotic regulation and cell wall synthesis. This led to inhibited or slowed mycelial growth and structural abnormalities in fungal hyphae. Conversely, the wild strain exhibited typical mycelial development patterns with vigorous growth and homogeneous pigmentation under optimal conditions. It displayed minimal signs of stress and maintained healthy hyphal structures with well-defined morphology. The wild strain demonstrated tolerance to fludioxonil and environmental stressors by retaining consistent pigment distribution and growth patterns across fungal colonies. The distinctions between the two strains highlight how crucial it is to understand how each strain reacts differently to environmental disturbances and antifungal medications. The wild strain maintained consistent growth and pigment synthesis, whereas the EC_{100} strain showed vulnerability to fludioxonil-induced stress, leading to altered morphology and disrupted pigmentation.

Alternaria alternata soluble protein content

The investigation aimed to examine the relationship between protein concentration and absorbance values by constructing a standard curve between the two variables. The relationship between the protein standard concentration (x-axis) and the corresponding absorbance values (y-axis) derived from the spectrophotometric measurements is described by the equation $y = 2.9233x + 0.0129$ (as shown in Fig. 4). The line's slope, or coefficient 2.9233, indicates the rate at which absorbance increases with increasing protein concentration. This slope value indicates a high degree of assay sensitivity, meaning that even minor changes in protein concentration cause detectable changes in absorbance measurements. At zero protein concentration, the absorbance value is represented by the y-intercept of 0.01290, which is probably caused by background noise or non-specific absorption.

Furthermore, the linear relationship between the protein concentrations and absorbance values accounts for 99.38% of the variance in the absorbance values, according to the coefficient of determination R^2 value. Across every range of protein concentrations examined, the high R^2 value indicated a good fit of the linear regression model to the data points, indicating excellent agreement between the observed and predicted absorbance values. As a result, the protein quantification assay's accuracy and reliability were confirmed by the linear equation and high R^2 value, demonstrating in the assay's capacity to accurately determine protein concentrations in unknown samples within the tested range. Additionally, the control protein ratios (CPr) of the wild and resistant strains were quantified. They were 0.192 and 0.187, respectively.

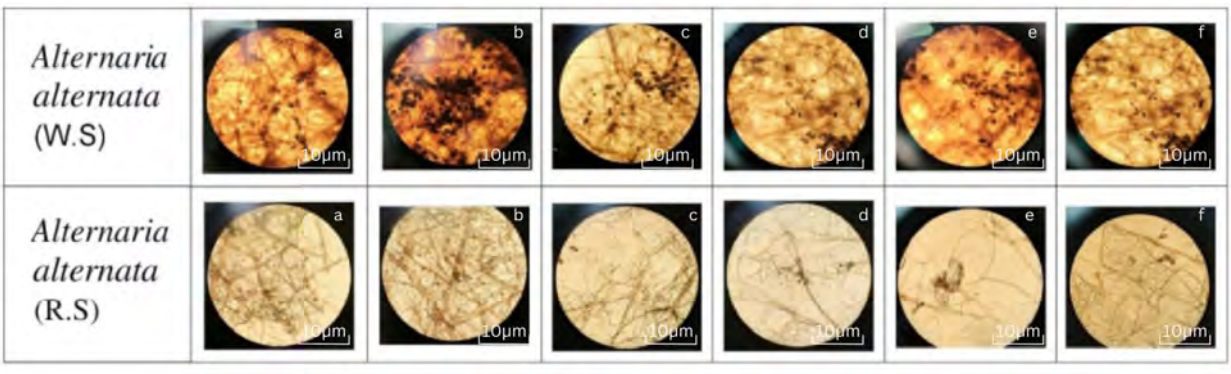

Fig. 3. The morphological differences between the wild strain (WS) and resistant strain (RS) of *Alternaria alternata*

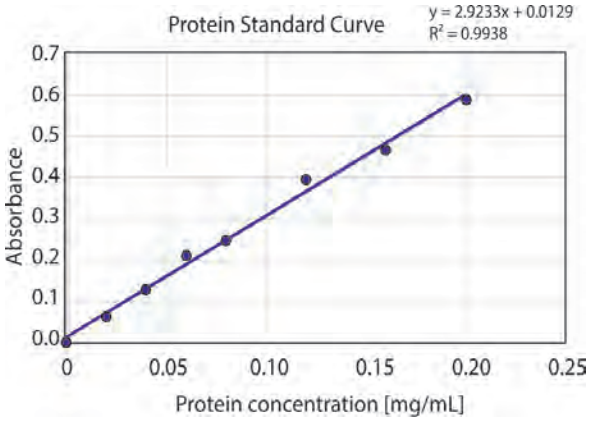

Fig. 4. The standard curve for the soluble protein assay showing the strong correlation between protein concentration and absorbance

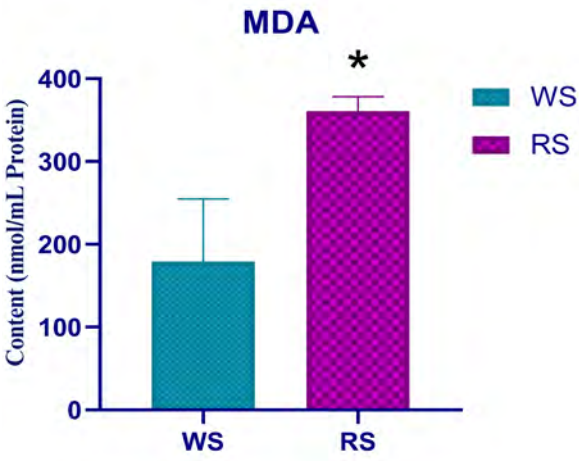

Fig. 5. Malondialdehyde (MDA) content of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

Variations in protein expression levels, enzyme activity, or metabolic processes may be indicated by differences in CPr values among strains. The resistant strain's greater CPr value in comparison to the wild strain may indicate increased enzymatic activity or higher expression of relevant proteins.

Malondialdehyde (MDA) content

There was a significant difference in malondialdehyde (MDA) content between the wild and resistant strains. Specifically, the data indicated that the resistant strain had higher MDA content than the wild strain. This finding suggests a significant difference in the oxidative stress response mechanisms between the two strains, with the resistant strain exhibiting higher MDA content, indicating that the resistant strain may be more susceptible to lipid peroxidation (as shown in Fig. 5). The resistant strain exhibited greater overall lipid peroxidation than the wild strain, based on its higher MDA content. Lipid peroxidation produces MDA as a byproduct and elevated MDA content signifies more oxidative damage to cell membranes. The resistant strain's observed mean MDA content of 360.89 nmol · mg^{-1} stands in sharp contrast to the wild strain's 179.19 nmol mg^{-1}. The resistant strain's low standard deviation of 14.13 indicates closely clustered MDA values around its mean, which was indicated by a high degree of consistency. On the other hand, the wild strain's higher standard deviation of 61.82 indicated greater variability in MDA content values. Compared to the wild strain, the resistant strain consistently showed greater MDA content, indicating enhanced oxidative stress and lipid peroxidation. Greater MDA content in the resistant strain may indicate increased oxidative stress or a distinct response to external stress factors (as shown in Table 2).

Table 2. Comparison of antioxidant enzyme activity between different strain types (wild type, drug-resistant type)

Enzyme activity type	Strain type	Catalase (CAT) U · ml^{-1} protein	Superoxide dismutase (SOD) U · ml^{-1} protein	Malondialdehyde (MDA) nmol · mg^{-1} protein	Tyrosinase activity U · ml^{-1} protein
Biological Replicate 1	wild type	12.678	165.509	144.262	22.8952
	resistant type	36.811	175.473	361.144	51.21241
Biological Replicate 2	wild type	12.725	148.463	266.062	23.64586
	resistant type	34.535	176.004	378.058	51.94012
Biological Replicate 3	wild type	10.330	170.107	127.254	25.14718
	resistant type	35.261	187.330	343.455	53.39553
Mean ± Std. Dev.	wild type	11.91 ± 1.12	161.36 ± 9.31	179.19 ± 61.82	23.90 ± 1.15
	resistant type	35.54 ± 0.95	179.60 ± 5.47	360.89 ± 14.13	52.18 ± 1.11
P-value		<0.01	0.12	0.002	0.001

Catalase (CAT) activity

The findings of this investigation showed that the wild and resistant strains differed significantly in catalase (CAT) activity. Specifically, the results indicated that the resistant strain had higher CAT activity than the wild strain. This observable difference highlights a significant difference in the enzymatic antioxidative capabilities of the two strains, with the resistant strain displaying higher CAT activity. This finding points to a possible increase in the resistance strain's capacity to reduce oxidative stress in cells by improving the catalytic hydrolysis of hydrogen peroxide (as shown in Fig. 6). The resistant strain appeared to have more catalase enzyme activity than the wild strain, based on its increased CAT activity. Higher CAT activity is indicative of a more effective antioxidant defense system. Catalase is an enzyme that is essential in the decomposition of hydrogen peroxide into water and oxygen. The resistant strain had a mean CAT activity of 35.54 U · ml^{-1} protein, whereas the wild strain's was significantly lower at 11.91 U · ml^{-1} protein. Furthermore, the resistant strain had a lower standard deviation (0.95) than the wild strain, which displayed a greater standard deviation (1.12). This discrepancy shows that the resistance strain's CAT activity values were more densely clustered, while the wild strain's values were more widely distributed around the mean. A greater standard deviation signifies that the data points within a dataset are more widely distributed relative to the mean. These findings suggest that, by consistently exhibiting higher CAT activity than the wild strain, the resistant strain may have had a more effective antioxidative defense mechanism (as shown in Table 2).

Superoxide dismutase (SOD) activity

The results of this investigation demonstrated that the wild and resistant strains differed significantly in terms of superoxide dismutase (SOD) activity. More specifically, the resistant strain had significantly higher SOD activity than the wild strain. This highlights a significant difference in the antioxidant defense systems functioning in the two strains, with the resistant strain exhibiting higher SOD activity. This finding suggests that the resistant strain possesses an enhanced ability to combat oxidative stress by effectively scavenging superoxide radicals, reflecting its enhanced enzymatic antioxidative response (as shown in Fig. 7). The resistant strain's increased SOD activity indicates that, on average, it had more superoxide dismutase enzyme activity than the wild strain. An effective antioxidant defense system was indicated by a higher level of SOD activity. SOD is an enzyme that is essential for neutralizing superoxide radicals. The resistant strain's mean SOD activity, measured at 179.60 U · ml^{-1} protein, significantly higher than that of the wild strain, which was measured at 161.36 U · ml^{-1} protein. The wild strain's greater variability standard deviation of 9.31 indicates greater variability in SOD activity measurements. The resistant strain, on the other hand, had a smaller standard deviation of 5.47, indicating that the SOD activity values were more closely clustered around the 179.60 U · ml^{-1} protein mean concentration. When compared to the wild strain, the resistant strain consistently exhibited greater SOD activity, suggesting a potentially more effective antioxidative defense mechanism (as shown in Table.2).

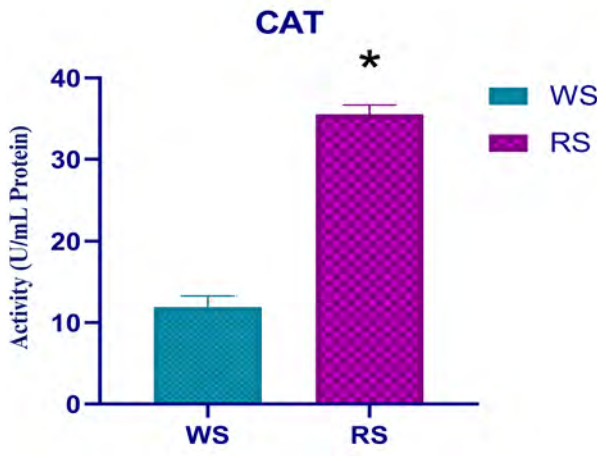

Fig. 6. Catalase (CAT) activity of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

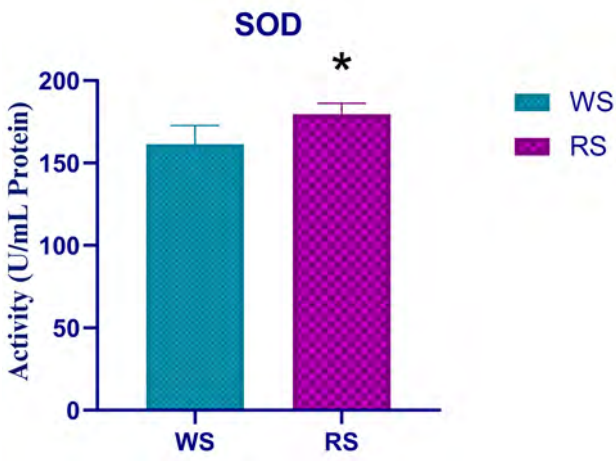

Fig. 7. Superoxide dismutase (SOD) activity of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

Tyrosinase activity

The results demonstrated significant differences in the tyrosinase activity of *A. alternata* wild and resistant strains. Specifically the tyrosinase activity of the resistant strain was significantly higher than that of the wild strain. This notable variation suggests a major difference in the melanin biosynthesis pathways of the two strains, with the resistant strain exhibiting increased tyrosinase activity. This finding suggests that the resistant strain has a higher melanin production capacity, which may help explain why it is greater resilience to environmental stresses. The resistant strain has greater tyrosinase activity than the wild strain, which suggests that the mean enzyme activity is higher. The resistant strain had a significantly higher mean activity of 52.18 ± 1.11 tyrosinase than the wild strain, which had a mean activity of 23.90 ± 1.15. Elevated melanin production, which is involved in defense mechanisms against UV radiation and oxidative stress, is associated with this increased tyrosinase activity. The resistant strain's tyrosinase activity values were more closely clustered around the mean of 52.18 U · ml^{-1} protein, as reflected by its lower standard deviation of 1.11. On the other hand, the wild strain's 1.15 standard deviation indicates that its tyrosinase activity values were slightly greater variability. Compared to the wild strain, the resistant strain exhibited a more robust enzymatic response under stress conditions, as seen by its consistently higher and more stable tyrosinase activity (as shown in Fig. 8).

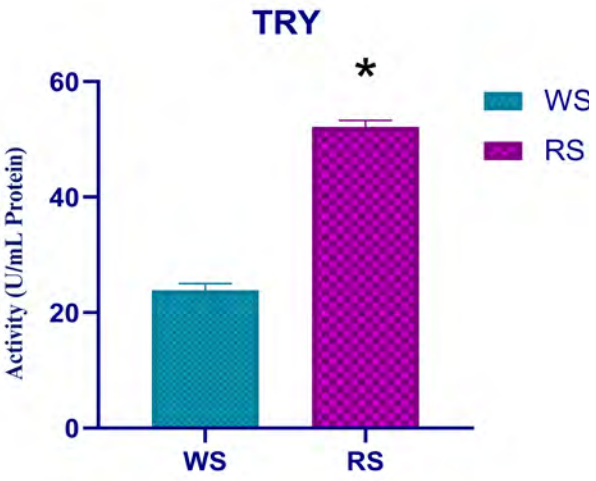

Fig. 8. Tyrosinase activity of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

Poplar pathogenicity

This experiment was conducted to evaluate the pathogenicity of poplar leaves (*Populus nigra*) with diseases, such as *A. alternata*. The treatment groups were as follows: CK (Control), which consisting of untreated leaves serving as a reference for comparison WS (Wild Strain), in which poplar leaves were inoculated with the wild-type strain of the pathogen, which is the naturally occurring form of the pathogen without any

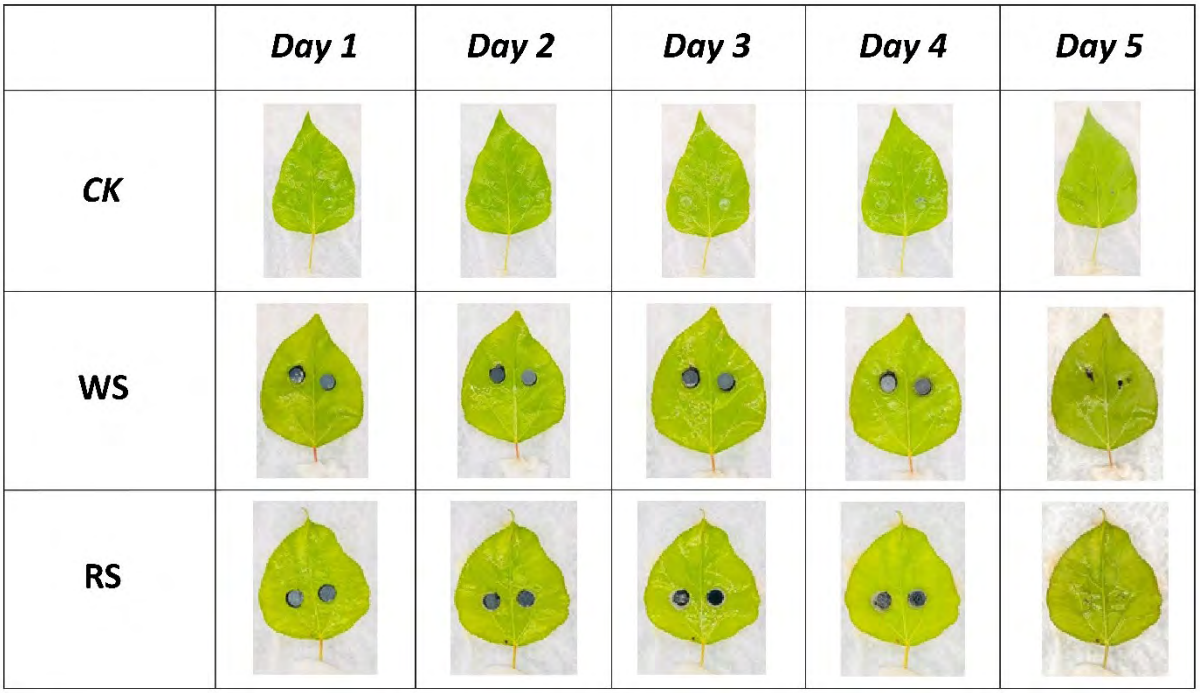

Fig. 9. Fludioxonil's effectiveness in reducing *Alternaria alternata* infection on poplar leaves, with minimal symptoms in resistant strain and disease progression in wild strain

alterations; and RS (Resistant Strain), in which poplar leaves were inoculated with a resistant strain of the pathogen that has become resistant to fludioxonil, potentially making it less virulent than the wild strain. The results showed that, throughout the incubation period, the CK leaves stayed healthy, suggesting that the pathogen was either absent or no disease developed in the absence of inoculation**.** Clear disease progression was observed in the WS leaves. Small lesions began to appear on Day 1. The lesions had increased in size and number on Day 2. On the third day, the lesions continued to expand and coalesce. The leaves showed significant damage with extensive necrotic patches by Days 4 and 5, suggesting that the infection was actively disease progression. There were few or no disease symptoms on the RS leaves. On Days 1 or 2, only a few minor lesions were observed, but they did not progress significantly. The leaves remained comparatively healthy by Days 4 and 5, suggesting that the resistant strain was exhibited reduced pathogenicity (as shown in Fig. 9).

Discussion

The growth of the *A. alternata* on the PDA plates was significantly inhibited by all fungicides (Feng and Zheng 2007) that have resistance against *A. alternata* at concentrations of 100 parts per million (ppm), 150 ppm, 200 ppm, 250 ppm, and 300 ppm. The fungicides and their concentrations had an impact on the rate of inhibition. The percentage of fungal inhibition increased as the increasing fungicide concentrations and the highest concentration tested resulted in the greatest degree of inhibition (Wang *et al.* 2023). The findings showed that different media types influenced *A. alternata* growth rates. As shown in Fig. 1, different types of media facilitated the fungal strain's growth. In all tested media, *A. alternata* mean radial growth differed significantly. Fungus showed the greatest degree of radial growth on PDA. Given that PDA is a conventional media with no internal resistance to *A. alternata*, this observation is consistent with predictions. On the other hand, different growth patterns were seen in media with resistance substances against *A. alternata*. In comparison to other treated media, RS-WM and CK showed the highest rates of growth. They showed that the growth conditions were ideal under such conditions. The existence of resistance elements is probably the reason why the EC_{100} medium did not grow as well as the CK media. Furthermore, RS-WM media showed greater growth than EC_{100} media, suggesting a possible benefit from the resistance characteristics in this strain. The wild strain's inoculation on resistant media may have had inhibitory effects, as the WS-RM exhibited limited growth in comparison to other tested media.

The most frequently isolated species from contaminated tomato fruits were *Alternaria* spp. (Zenelt *et al*. 2021; Schmey *et al*. 2023; Biswal and Das 2024). Using the PDA medium, 20 *Alternaria* isolates were described both macro- and microscopically, leading to the classification of these isolates into five species: *A. alternata, A. brassicicola, A. citri*, *A. radicina*, and *A. tenuissima*. The classification of the five isolated morphological types of *Alternaria* spp was made possible by varied colony morphological differentiation observed on the PDA and by microscopic inspection (El-Ganainy *et al*. 2021; Saleem and El-Shahir 2022). It was shown that the wild type and fludioxonil-resistant EC_{100} strain of *A. alternata* differed significantly. Because of fludioxonil interference, the EC_{100} strain exhibited reduced mycelial growth, collapsed mycelium, and uneven coloration. Under optimum conditions, the wild strain exhibits solid morphology, uniform pigmentation, rapid growth as well as hyphal resilience by maintaining uniform growth and pigment dispersion during stress or exposure to fludioxonil.

The soluble protein content of the mycelium was significantly reduced by both copper (II) hydroxide wettable powder (Cu $(OH)_2$ WP) and copper-based nanomaterials (Cu-based NMs), with dose-dependent differences being visible (Yuan *et al*. 2023; Parada *et al*. 2024). A common indicator of the degree of cell damage in eukaryotes is the concentration of specific soluble proteins, which can arise under a variety of stressful circumstances (Li Jiao *et al*. 2018). An *A. alternata* infection affected the malondialdehyde (MDA) level in mango fruit. The MDA contents of *A. alternata*-infected mango fruit were significantly higher than those of the untreated control fruit, which might be explained by the significant lipid oxidation of MDA that occurs in response to *A. alternata* infections (Li Jiao *et al*. 2018; Peić Tukuljac *et al*. 2023). Our results showed that a noteworthy distinction in MDA content between the resistant and wild strains. In more exact terms, the results suggest that the resistance strain exhibits more MDA contents than the wild strain. This result indicates a notable distinction in the two strains' oxidative stress response systems, with the resistant strain showing higher MDA contents and possibly being more vulnerable to lipid peroxidation (Fig. 5). Based on its greater MDA contents, the resistant strain appeared to exhibit more lipid peroxidation than the wild strain.

The plant defensive systems against many stresses, including pathogen invasion, are impacted by the enzymes ascorbate peroxidase (APX), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), POD, SOD, and CAT (He *et al*. 2017; Wiraswati *et al.*

2019). Superoxide and hydrogen peroxide radicals are changed into less dangerous and more stable forms by the detoxifying enzymes CAT, SOD, and peroxidases, which deal with ROS (Hossain *et al*. 2015). The first line of defense is CAT and peroxidases, which further SOD-catalyzed dismutation of superoxide anion into oxygen and hydrogen peroxide. Hydrogen peroxide removal involves members of a large class of enzymes known as peroxidases (Ihsanullah *et al*. 2024; Jomova *et al*. 2024). The primary distinction between them is the kind of reducing substrate that each one utilizes: pyrogallol peroxidase uses pyrogallol, guaiacol peroxidase uses guaiacol, and ascorbate peroxidase uses ascorbate (Hiner *et al*. 2000; Morales-Urrea *et al*. 2023). Increases in the activities of CAT, SOD, and peroxidase suggest that the initial infection stimulated these enzymes, which in turn activated the antioxidant defense system of the plant (Hernández *et al*. 2016). Compared to control fruits, the inoculated fruits of Una and Kurtovska kapia exhibited reduced CAT activity. This aligns with the findings of Li Jiao *et al*. (2018). CAT activity in mango fruits infected with *A. alternata* decreased drop-by-drop. After Kurtovska kapia fruits were injected with water, the level of CAT activity was maximum. Amfora's CAT activity was much lower than that of the control group when water was put into the fruits. There is a decrease in CAT and SOD levels in avocado fruit during wound stress (Jiao *et al*. 2018; Muhammad *et al*. 2024). The conclusions from this investigation demonstrate that there were notable differences in the CAT activity of the normal and resistant strains. Specifically, the investigation showed that the resistant strain exhibited greater CAT activity than the normal strain. The resistant strain had increased CAT activity, highlighting a discernible difference in the two strains' enzymatic antioxidative capabilities. This result suggests that the resistant strain may have been better able to catalyze the hydrolysis of hydrogen peroxide, thereby reducing oxidative stress in cells (Fig. 6). However, the results of this investigation conclusively demonstrated that there were notable differences in SOD activity between wild-type and resistant strains. More precisely, the findings indicated that the wild strain's SOD activity was substantially lower than that of the resistant strain. This notable distinction indicates that the two strains' antioxidant defense mechanisms operated very differently, with the resistant strain having greater SOD activity. This finding highlights the resistant strain's improved enzymatic antioxidative response (Fig. 7) and implies that it may be better able to fight oxidative stress by efficiently scavenging superoxide radicals.

Tyrosinase is a monooxygenase that contains copper and catalyzes the O-hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine and dopaquinone. In eukaryotes, this process is crucial for the formation of melanin (Maamoun *et al*. 2021). Tyrosinases are metalloenzymes with copper that are found in bacteria, fungi, plants, insects, and mammals. They are involved in a variety of biological activities (Khan *et al*. 2018; Beaumet *et al*. 2023). Tyrosinase is the main rate-limiting enzyme in the manufacture of L-DOPA melanin, which is widely found in microorganisms, plants, animals, and the human body (Rao *et al*. 2011; Ihsanullah *et al*. 2024). Although tyrosinase is a component of the L-DOPA melanin production pathway, its function in the pathophysiology of *M. oryzae* is still unclear. Biochemical, functional genetics, and cell biology methods were used to assess the role of the unidentified gene (MGG_14598) called tyrosinase (MoTyr) in the pathogenic and morphological development of the rice blast fungus, which is economically devastating. Many fungi have well-conserved orthologs of tyrosinase (Rao *et al*. 2011; Li *et al*. 2021a, b). Tyrosinase research in the medical, cosmetic, culinary, and other domains has been primarily focused in recent years; nevertheless, studies pertaining to tyrosinase in pathogenic fungi are lacking (Fan *et al*. 2023). Our finding showed that the tyrosinase activity of the resistant strain was significantly higher than that of the wild strain. This notable variation suggests a major difference in the routes by which the two strains produce melanin, with the resistant strain exhibiting increased tyrosinase activity (Fig. 8). This finding raises the possibility that the resistant strain has a higher melanin production capacity, which may help explain why it is more resilient to environmental stresses. The resistant strain had greater tyrosinase activity than the wild strain, which suggests that the mean enzyme activity was higher.

Neofusicoccum parvum and *Botryosphaeria dothodea* are both considered important fungal species in the Botryosphaeria genus of the Botryosphaeria family, according to research findings. *B. dothodea*, while previously thought of as a potentially dangerous fungus, is very common and cryptic in forestry, agricultural, and natural forest ecosystems, whereas *N. parvum* has been observed to cause cankers and diebacks in a variety of woody species worldwide (Marsberg *et al*. 2017; Khan *et al*. 2018; Hassan *et al*. 2021; Ihsanullah *et al*. 2024). The findings of our experiment research demonstrated that the absence of *A. alternata* illness and the continued health of the CK leaves. Lesions in the WS leaves started to be visible on Day 1 and grew by Day 3, resulting in significant damage by Day 5. By Day 5, the RS leaves looked largely healthy and displayed minor symptoms, including a few lesions that did not spread. This suggests that the sickness was less severe.

Conclusions

The resistance mechanisms of *A. alternata* to environmental stressors and fungicide resistance were investigated in this study. The main conclusions were that the resistant strain exhibited reduced mycelial development, altered pigmentation, and higher antioxidative enzyme activity (SOD, CAT, tyrosinase, and MDA) than the wild strain; fungal development was influenced by growth media, with notable variances in growth rates between media types. A significant challenge was the wild strain's inhibited growth on resistant media, underscoring the intricacy of managing fungal resistance. The resistant strain was found to inhibit the fungal effects on *Populus nigra* leaves, indicating its potential to mitigate plant damage. In light of these results, we advise more research into the molecular processes that underlie these reactions in order to create focused interventions that will enhance disease control and advance methods for sustainable agriculture.

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ORIGINAL ARTICLE

Ecological distribution patterns of *Eremina desertorum* in relation to *Zygophyllum album*, *Thymelaea hirsuta*, and climatic factors in its habitat in Egypt

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Abstract

This study examined the distribution of the desert snail *Eremina desertorum* (Forskål, 1775), a mollusk of economic importance, in arid desert environments. By exploring its ecological relationship with the plant species *Thymelaea hirsuta* (L.) Endl. and *Zygophyllum album* L.f. in Egypt, the research highlighted the correlation between these plants and the snail's habitat preferences. Over the course of a year (from March 2021 to March 2022), 40 field visits were conducted across various locations along Egypt's Mediterranean coast, encompassing all seasons. It was observed that *E. desertorum* tended to aggregate on *T. hirsuta* and *Z. album*, rather than on other wild plant species. Thus, the study aimed to predict the spatial distribution of *E. desertorum* by analyzing its relationship with these associated plants in Egypt, and the effect of prevailing climatic factors on its distribution, particularly seasonal precipitation and relative temperature. Spatial analyses over a decade (2012–2021) indicated that most *E. desertorum* populations were concentrated around the Nile and Upper Nile Deltaic regions, where *Z. album* was more prevalent than *T. hirsuta*. In contrast, *T. hirsuta* was predominantly recorded in the upper parts of Egypt, near the Mediterranean coast. Findings demonstrated a strong link between regions with higher precipitation and the presence of *E. desertorum* and its associated plants from the Zygophyllaceae (*Z. album*) and Thymelaeaceae (*T. hirsuta*) families. Furthermore, the snail showed a preference for plants known for higher water retention, which likely aids its survival in arid, water-scarce environments. These findings offer a useful framework for predicting the distribution of *E. desertorum* in relation to key plant associations and climatic conditions in arid environments. While the spatial data were based on previously recorded location coordinates, further studies focusing on population dynamics and broader plant comparisons could enrich the understanding of habitat preferences under shifting climate patterns.

Keywords: Egyptian flora, *Eremina desertorum,* precipitation. spatial analyses, temperature

Introduction

The phylum Mollusca is the second largest invertebrate phylum that has a global distribution (Atta *et al.* 2021). Terrestrial gastropods are the only mollusca class that has successfully invaded land which are widely dispersed by human activities, particularly by transporting soil and plant materials (Speiser and Kistler

2002). Terrestrial mollusks, including snails and slugs, belonging to the class Gastropoda, have significantly increased in their economic importance. Some species have become agricultural pests in the Nile Delta and occur in high numbers in the North Coast belt of the Mediterranean Sea (Mahmoud and Awad 2008; Mohammed 2015; Abdel kader *et al.* 2016). Additionally, Gastropoda represents a significant group of economic pests, exemplified by snails that pose a substantial threat to various agricultural and horticultural crops in temperate and humid environments across the globe. Variations in dispersal, movement, and daily activity patterns among land snail species are influenced by climatic conditions. Land snails feed on leaves, roots, tubers, and ornamental plants. Furthermore, they produce a mucus with an odor, which serves as a deterrent to humans and deters herbivory consumption of these contaminated plants (Khidr *et al.* 2020). The odor also impacts the population density and activity of land snails (Khidr *et al.* 2020). Land snails have been reported to damage tender plant tissues such as wheat blossoms, Egyptian clover, various vegetable crops, and fruits (Desoky 2018). They attack field crops, orchard trees, as well as ornamental and medical plants (El-Wakil *et al.* 2000; Abo-Elwfa *et al*. 2024). Snail slime contamination has led to animals avoiding feeding on contaminated plants. Consequently, the consumption value of these plants has diminished, resulting in a decline in crop marketability and export potential (Ali and Robinson 2020). In the geographical distribution correlation of snails with their plant habitats, the abundance density of snails decreased by increasing distances between orange trees (Khidr *et al.* 2020).

The bodies of many species of snails and slugs that belong to this phylum, such as *Eremina desertorum* (Forskål, 1775), secrete rich mucus which helps reduce desiccation under arid conditions (Gabriel *et al.* 2011; Nantarat *et al.* 2019; Ibrahim *et al.* 2022). This mucus and its derivatives, like mucin, may contribute to its beneficial pharmacological activities (Gabriel *et al.* 2011; Trapella *et al.* 2018; Abd El Azeem *et al.* 2020). For instance, it has been suggested that *E. desertorum* mucin holds potential as an antioxidant, hepatoprotective, and anti-inflammatory agent for the treatment of hepatic disorders as well as colon and liver cancers (Atta *et al.* 2021; El-Zawawy and Mona 2021; Ibrahim *et al.* 2022).

Eremina desertorum snails live in sandy deserts and feed on shrubs (Ali *et al.* 2016; Holyoak *et al.* 2018). Throughout the year desert snails occur on wild plants in a natural, arid ecosystem with low precipitation. (Ali 2017a, b). This desert species is commonly found across various locations along the Mediterranean region, from Alexandria to Egypt's border with Libya (Kaltenbach *et al*. 1934; Ali 2017a). Arad reported that *E. desertorum* has a high degree of resistance to water loss in standardized laboratory studies (Kaltenbach *et al*. 1942). Therefore, it can live in an arid desert environment, which feeds and finds shade under many wild plants (Holyoak *et al.* 2018). *Eremina* L. Pfeiffer, 1855 is a geographically restricted genus to many countries of the North Africa region (Schileyko 2000) such as Egypt, Libya and Tunis (Ali 2017b). Its distribution encompasses the entirety of the northern desert region of Egypt and the desert of northern Sinai, and southern Tunisia. Additionally, it can be found along the shores of the Red Sea and the Atlantic Ocean up to Rio of Oro, a southern geographic region located in northwestern Africa (Pallary 1909; Pallary 1924). The genus is also present in the southwestern regions of Morocco and Mauritania and apparently in the Cape Verde Islands (Groh and Rolán 2005; Groh 2012). The genus is unknown in E. Morocco and Algeria, extending southwards through Arabia to Somalia (Neubert 1998). *Eremina* samples were collected from many regions along Egypt's northern coast from Alexandria to El Sallum village (Ali *et al.* 2016). According to previous studies, the presence of these mollusk species in arid desert regions can be attributed to favorable and suitable environments that support their growth and reproduction. This is facilitated by the existence of certain wild desert plants.

Thymelaea hirsuta (L.) Endl. is the sole taxon within the Egyptian flora, classified as *Thymelaea*. The native range of this species encompasses the Mediterranean region. The plant in question is classified as a subshrub or shrub, and its primary habitat is the subtropical biome. Furthermore, it inhabits dunes, sand flats, rocky ground, banks of water bodies, plains, depressions, wadis, roadsides, and cultivated lands of oases, North Sinai, and the Mediterranean western desert of Egypt (Boulos 2000; Bedair *et al.* 2020). It is a prevalent species found in Egypt, serving as a habitat for a wide range of fauna throughout the year (Hegazy *et al.* 2022). The population of *T. hirsuta* in Egypt is experiencing a decline, resulting in habitat reduction potentially leading to species extinction in the future (Bedair *et al.* 2020). An almost continuous row of tourist facilities occupies the coastline along the northern coast. This has not only led to the complete destruction of the habitats, but also degradation of vast areas of habitats surrounding them. Consequently, this threatens plants in these habitats such as *T. hirsuta* (Książkiewicz-Parulska and Ablett 2016; Hegazy *et al.* 2022). *Zygophyllum album* L.f. is a prevalent subshrub characterized by its typical woody stem that supports slender branches adorned with succulent leaves. It thrives in the saline sandy soils of coastal and inland regions of the Mediterranean, Oases, Sinai, the Red Sea, and Egyptian deserts (Boulos 2000). The native range of this species globally is near-endemic (NE) Spain, S. & E. Mediterranean, the Arabian Peninsula, and Somalia. It grows primarily

in a subtropical biome (POWO 2021). Interestingly, this species serves as a microhabitat for several snail species during the summer (Aubry and Magnin 2005).

Accordingly, climate changes over the years may alter the chemical composition of certain weeds, which in turn can affect the organisms that feed on them. Temperature fluctuations have influenced the life traits of organisms, which eventually have dictated population growth rates (Nikolova 2025). Indeed, predicting how different species will respond to environmental changes is challenging due to the diversity of natural ecosystems (Balakrishnan and Yapp 2004). Understanding the distribution patterns of species within their habitats is crucial for ecological research and conservation efforts (Balakrishnan and Yapp 2004). In arid environments like Egypt, where desert ecosystems prevail, investigating how organisms adapt to their surroundings becomes particularly relevant. In this study, the distribution patterns of the desert snail *E. desertorum* in its natural habitat were focused on. Specifically, its interactions with two common plant species, *Z. album* and *T. hirsuta,* were explored. By modeling these distribution patterns, the aim of this study was to better understand the ecological dynamics and adaptations of this snail species under the challenging desert conditions of Egypt. The study aimed to investigate the predictive occurrence of the geographic range of the interaction between *E. desertorum* and two plant species, *Z. album* and *T. hirsuta.* Using field observations of this snail on the plants under study in its habitat in Egypt the study sought to explore the influence of climatic changes on the distribution of the desert snail in conjunction with the two desert plants through spatial analyses.

Materials and Methods

Study Area

Egypt is situated in the northeastern part of Africa and includes the Nile region, which encompasses temperate grassland, desert, and semi-desert biomes. The Nile region in Egypt spans around 1,520 kilometers, which accounts for 23% of the entire length of the river and sustains a population of approximately 80 million individuals. The composition of the region includes three primary elements: the Nile Delta, Nile Fayium, and Nile Valley. The Nile Delta, along the Mediterranean coast, extends 240 km from Alexandria to Port Said. The Nile Fayium, a wind-eroded depression formed about 1.8 million years ago, covers approximately 12,000 square kilometers and lies below sea level (Ammar 2022) (Fig. 1). The Nile Valley and the southern part of the Nile Delta are hyper-arid, while the northern section is arid, reflecting Egypt's overall arid to hyper-arid climate. From 2012 to 2018, annual rainfall varied between 80 and 200 mm, with the Mediterranean coast near Alexandria receiving the most precipitation. The hot and arid summer season lasts from May to October (https://weather-and-climate.com). This study relied on the fact that the desert snail *Eremina desertorum* was observed in abundance on certain desert plants, *Thymelaea hirsuta* and *Zygophyllum album*, in its habitat in Egypt, where it was observed over the course of a year (from March 2021 to March 2022), during 40 field visits. Observations along the Mediterranean coast, were conducted over all seasons. It was seen that *E. desertorum* tended to congregate on these

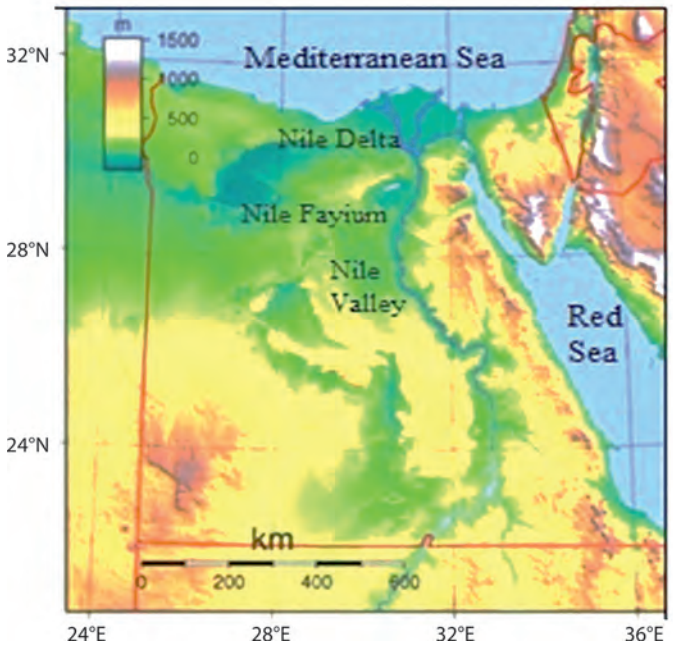

Fig. 1. Topographical map of the Nile region in Egypt, illustrating the varying landscapes and geographical features within the area

plants more than on others. Therefore, it was necessary to elucidate the geographical distribution of the desert snail on some associated desert plants in Egypt, and the effect of prevailing climatic factors on its distribution over a decade-long period spanning from 2012 to 2021.

Eremina desertorum occurrence datasets

The recorded locations of *E. desertorum* (from the year 2000 until now) were compiled through access to available online databases and literature searches. Databases were used to search for any literature related to the occurrence and distribution of the land snail *E. desertorum* and its above-mentioned sub-species. The obtained search results were examined to extract literature that recorded this snail in Egypt during the study period. Accordingly, a distributional data set was created that included geographical coordinates. If the geographical coordinates were not available for a region, we assigned them through geographical maps. The recorded locations and geographic coordinates of *E. desertorum* in Egypt (El-Wakil *et al.* 2000; Hassan 2015; Ali 2017a; Ali *et al.* 2016; El-Zawawy and Mona 2021) are shown in Table S1. This species was formerly known as *Helix desertorum* Forskål, 1775.

Plants occurrence datasets

Regarding *T. hirsuta* and *Z. album*, the available data on locations and distributions of the studied plant species were recorded, from March 2021 to March 2022, including all the seasons. Different locations were visited which covered the phytogeographical regions of the Oases, the Mediterranean, the Nile region, the Red Sea, the Eastern desert and Sinai and from the herbaria of Tanta University (TANE), Cairo University (CAI), Assiut University (ASTU), Agricultural Research Center (CAIM), Desert Research Center (CAIH), National Research Centre (CAIRC), and National Registry for Egyptian Herbaria (2023). These recorded locations are shown in Table S2.

Geospatial and statistical data analyses

A geospatial distribution map was created to demonstrate the relationship between localities of *E. desertorum* (both empty shells and living samples) and the geographical distribution of both *Z. album* and *T. hirsuta*. Point feature classes (for both plant and animal localities) were created in ArcMap 10.9 (Law and Collins 2015). Additionally, all vector shapefiles were spatially projected and geo-referenced to the World Geodetic System (WGS) 1984 datum. For plant and animal localities, annual averages for both precipitation ($mm \cdot day^{-1}$) and temperature (°C) were presented during the years 2013, 2017, and 2021 as graduated color classes overlaid on top of the point feature classes for both animal and plant locations. To test the significance ($p < 0.05$) of the relationship between precipitation ($mm \cdot day^{-1}$) and temperature (°C), Pearson correlation coefficients (r) were calculated. All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, U.S.A.).

This study investigated the influence of temperature and precipitation on the occurrence of *E. desertorum.* Data on temperature and precipitation were collected for 10 years, from 2012 to 2021, utilizing the Power NASA website (https://power.larc.nasa.gov/). Subsequently, the relationship between them was tested (Tables S1 and S2). Occurrence records from different periods were combined for spatial distribution analyses.

Results

The occurrence and distribution of terrestrial gastropods vary significantly depending on habitats, vegetation cover and climatic conditions. This variation is evident because many of these environmental variables provide favorable habitats for these animals. Regarding the taxonomic and descriptive background of the desert snail, the desert snails *E. desertorum* (Forskål, 1775) from the Helicidae family (Gastropoda: Pulmonata) are terrestrial snails in the genus *Eremina*. The shell of this genus ranges from 14 to 26 mm in height, with 22–35 mm in diameter (Blume 1952). It has a semi-spherical shell which is quite solid, opaque with shouldered whorls, and white to yellowish-corneous. There are reddish bands or yellowish streaks with irregular radial striation. The aperture is round, sometimes slightly angulated, straight or with a little reflexed margin. The umbilicus is very small or absent (Ali 2017b).

The size of the shells of this genus ranges from medium to large, depressed with thick walls (Neubert 1998). Shell size and shape are related to climate change from cooler and more humid conditions along the Mediterranean coast to arid and hot conditions inland (Ali *et al.* 2016). This genus is represented by a number of species, including sub-species *E. desertorum desertorum* (Forskål), *E. d. irregularis* (Férussac) and *E. d. zitteli* Boettger, which are reported in northern Egypt (Ali 2017a).

Eremina desertorum and its habitat plants' geographical distribution

The desert snails were observed to congregate more frequently on the wild plants *Zygophyllum album* L.f. and *Thymelaea hirsuta* (L.) Endl. during field trips conducted from March 2021 to March 2022, across

the study area. These trips covered all four seasons, including dry, wet, hot, and cold conditions (Fig. 2). Therefore, the relationship between the geographical distribution of both snails and plants was studied to understand the extent of the correlation between their distribution. After analyzing the plants' geographical distribution at the recorded sites (Fig. 3), it was observed that the predominant occurrence of *Z. album* was concentrated along the Mediterranean Sea coastline, except for three specific locations situated on the southern region of the Red Sea coast (Wadi Gimal, 50 km south Marsa Alam, and 12 km north Marsa Alam, see Table S1 for exact site location). In the Nile Delta (Fig. 4A), *Z. album* was recorded at two sites. In contrast, *T. hirsuta* was exclusively recorded in the northern region of Egypt, predominantly in close proximity to the Mediterranean Sea coastline.

The analysis conducted on the geographical distribution of *E. desertorum* revealed that a significant majority, more than 90% of the observed localities, were found close to the Nile River watershed areas and in the upper regions of the Nile Delta (Fig. 4C). Both empty shells and living samples of *E. desertorum* were recorded near the locations of both *Z. album* and *T. hirsuta* (see field images presented in Fig. 2 for more details). It was noted that the distribution of

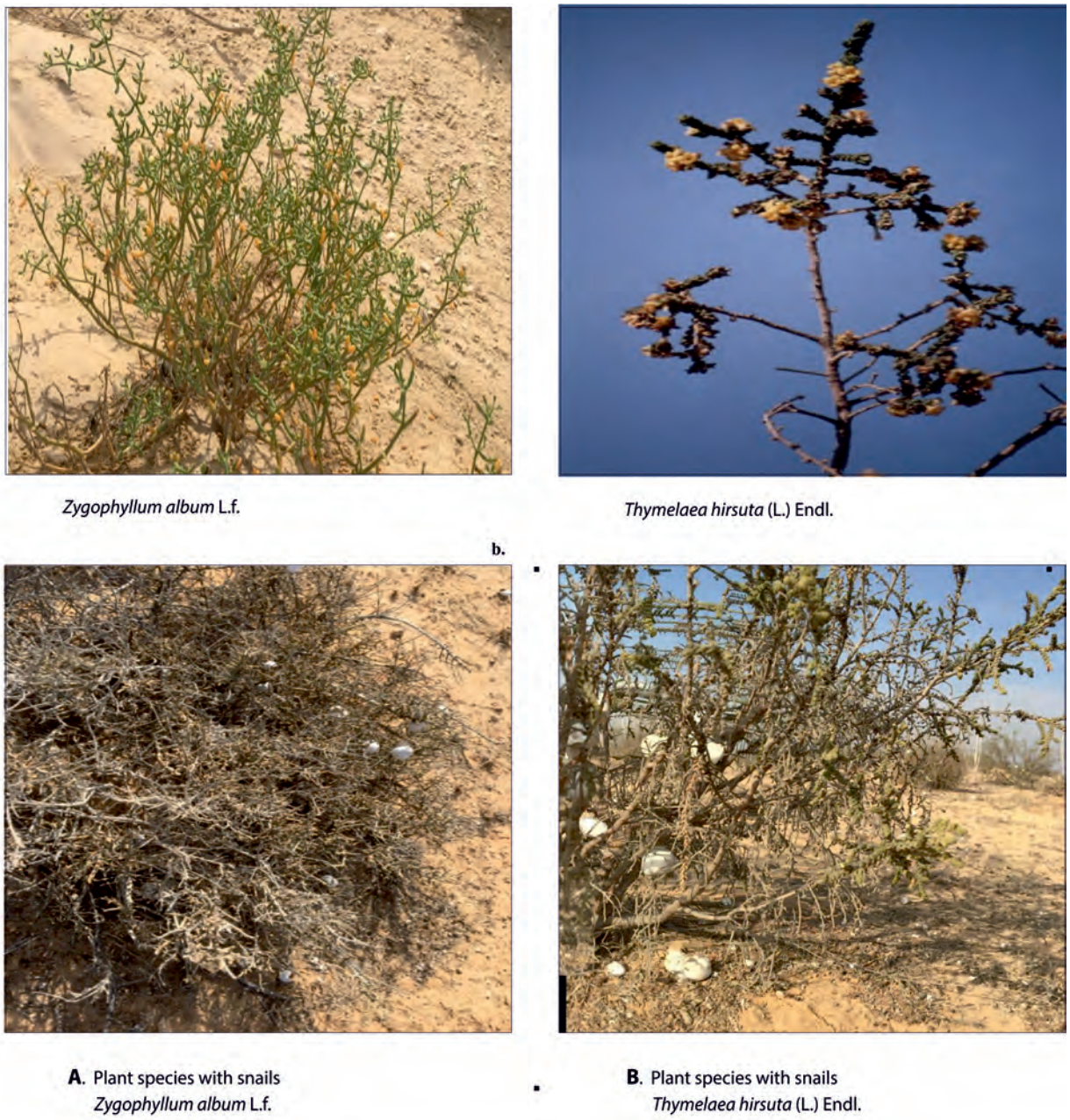

Fig. 2. *Eremina desertorum* snails found on *Zygophyllum album* L.f. – A and *Thymelaea hirsuta* (L.) Endl.– B along the Mediterranean Sea coast of Egypt (photo courtesy of the authors)

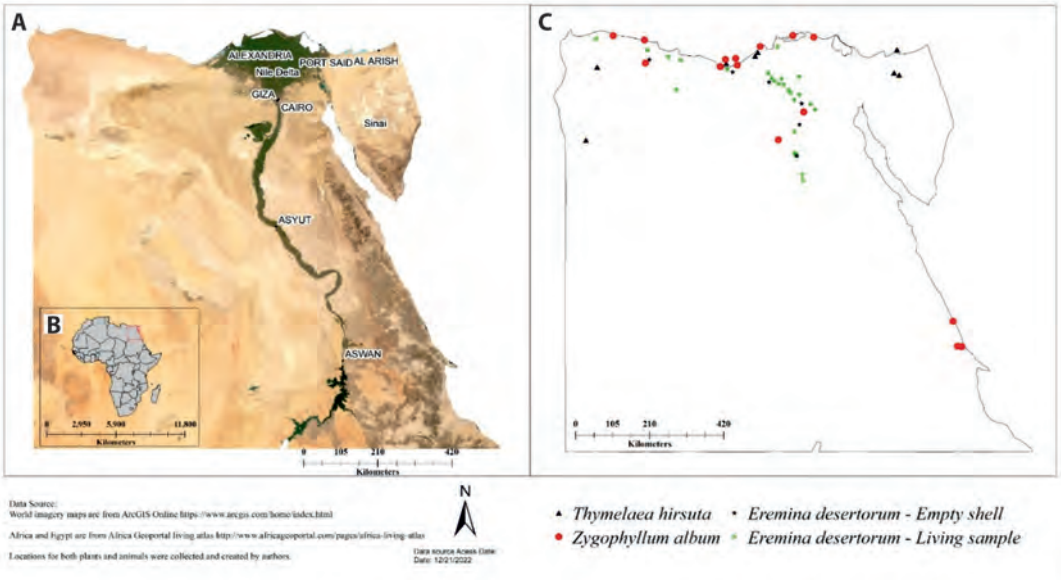

Fig. 3. A – Geographical distribution map of study sites in Egypt, displaying the locations of plant species (*Zygophyllum album* and *Thymelaea hirsuta*) in relation to *Eremina desertorum*. B – The inset map highlights the boundaries of Egypt within Africa. C – map generated using ArcMap 10.9.

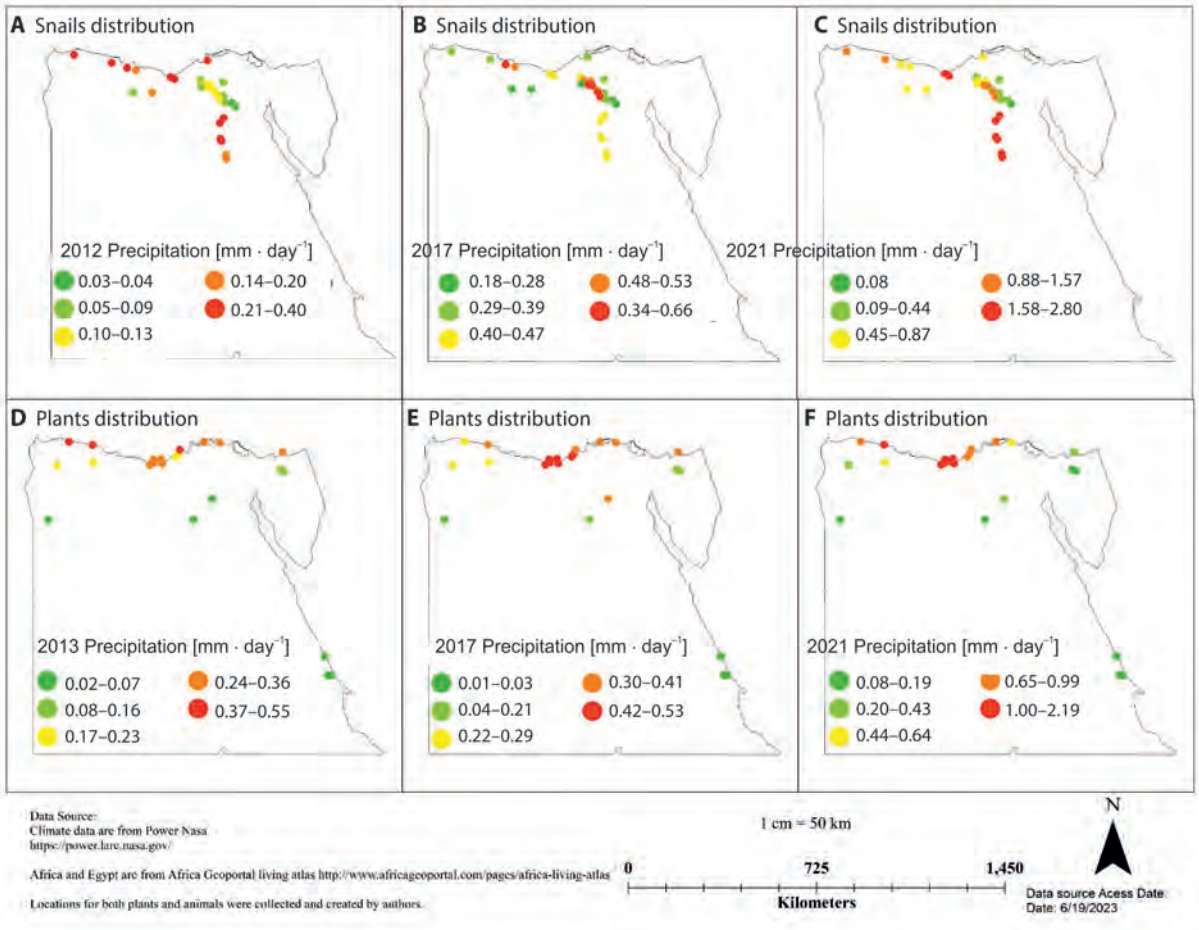

Fig. 4. Geographical map illustrating variations in precipitation (mm · day^{-1}) from 2012 to 2021, in relation to the distribution of *Eremina desertorum* snails and the plants *Zygophyllum album* and *Thymelaea hirsuta*. Map created using ArcMap 10.9

E. desertorum was more associated with *Z. album* than *T. hirsuta*, as indicated in the geographical distribution map (Fig. 4). For *E. desertorum* distribution, only six locations (out of 33) were noted as empty shells (Fig. 4C).

Climatic factors impacting plant and snail geographical distribution

The results of this research revealed that the potential distribution of snails associated with *T. hirsuta* was concentrated in the Mediterranean region in Omayed, Al-Alamein, Sallum and Lake Mariut, and the northern Sinai mountains of Halal and Maghara. *Z. album* was distributed on the Mediterranean in Burrulus, Al-Alamein, Burg El-Arab and Maqtala, the Red Sea in Wadi Gimal and Mersa Alam, the Eastern Desert in Wadi Degla and the Cairo-Suez Road, and the Nile Fayium. Annual averages for both precipitation (mm day-1) and temperature (°C) were presented along with the geographical distribution of snail and plant localities (Fig. 4 and 5, respectively) during 2013, 2017, and 2021 using the Power Nasa website (https://power.larc.nasa.gov/). Overall, for both snail and plant localities, higher precipitation was noted near the Mediterranean coast and the Nile Delta from 2012 to 2021 (Fig. 5). When the exact locations were compared, it was noted that there was higher precipitation (mm day^{-1}) during 2021 than in 2012 or 2013 (Fig. 5). For example, the highest precipitation (mm day^{-1}) was recorded during 2021 and ranged from 1.58 to 2.80 mm day-1 for snails' localities (Fig. 4 C) and 1.0–2.19 mm · day^{-1} for plant localities (Fig. 5 F) near the Mediterranean coast. Conversely, for the same snail and plant localities, 2012 had the lowest precipitation (mm · day^{-1}).

In 2017, the precipitation in the snail localities located south of the Deltaic region (Fig. 4B) was observed to be significantly lower than the precipitation levels recorded in 2012 (Fig. 4A) or 2021 (Fig. 4C). For temperature (°C) changes in terms of snail and plant geographical distribution, analyses of the

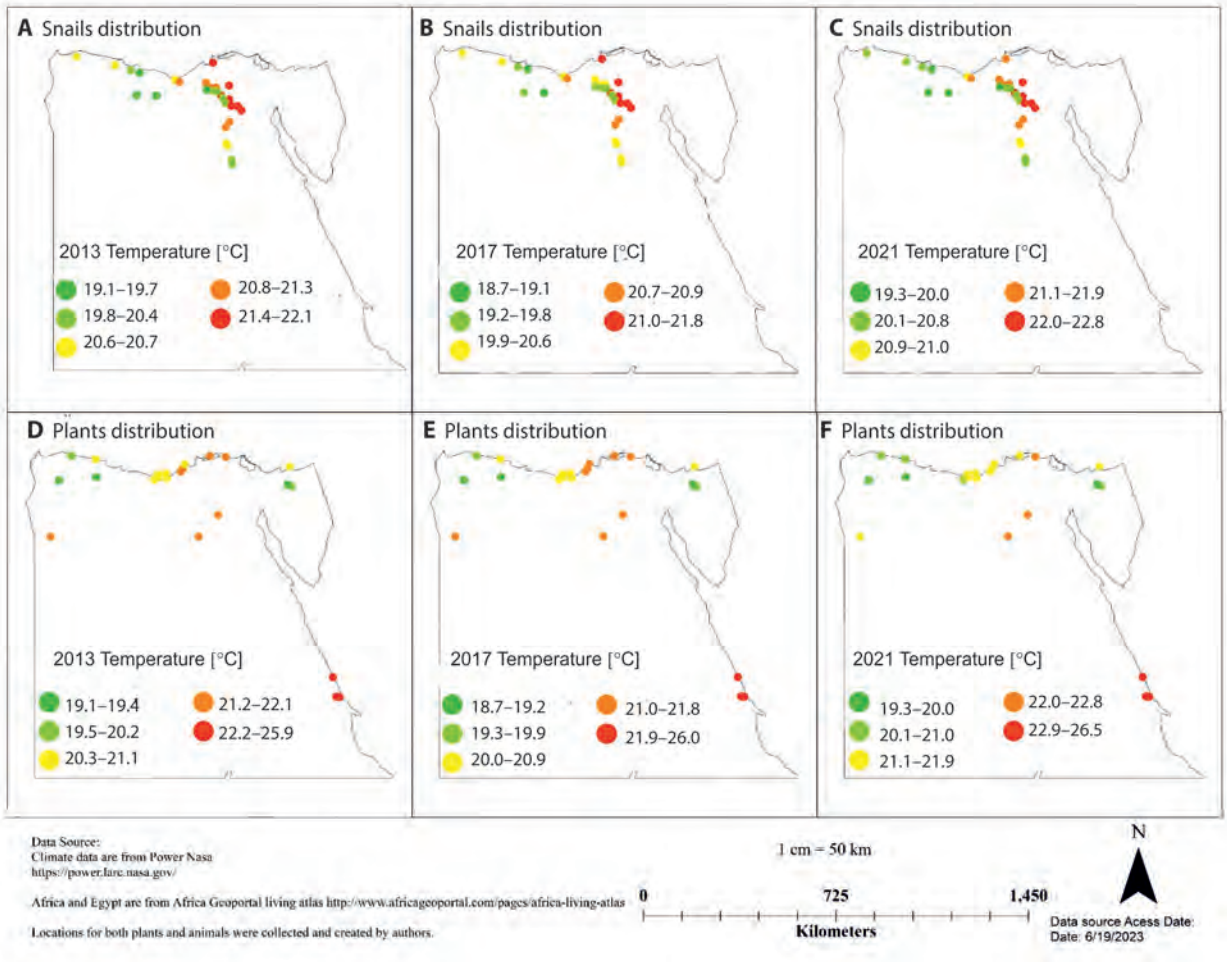

Fig. 5. Geographical map displaying the variations in air temperature (°C) from 2012 to 2021, in relation to the distribution of *Eremina desertorum* snails and the plants *Zygophyllum album* and *Thymelaea hirsuta*. Map generated using ArcMap 10.9

presented results indicated that coastal areas noted higher changes in temperature (°C) than inland areas (Deltaic and deserts areas, Fig. 5). In the context of snail geographical distribution and its correlation with temperature records spanning from 2012 to 2021, it was observed that there was a general increase in air temperature. Specifically, the lowest recorded temperature in 2012 was 21.0°C (Fig. 5A and Fig. 5B), while the highest temperature was recorded in 2021 at 22.8°C, (Fig. 5C) when comparing precise locations within Deltaic regions. The temperature ranges observed in plant locations, particularly in the southern region of the Red Sea (as depicted in Fig. 5D, 5E, and 5F), were notably higher. These locations include Wadi Gimal, located 50 km south of Marsa Alam, and two locations near Marsa Alam, one located 12 km to the north. It is worth noting that Z. album was documented in these specific areas. Statistical analyses revealed a significant negative correlation ($r = -0.4$, $p < 0.05$) between precipitation ($mm \cdot day^{-1}$) and air temperature (°C). The above finding was additionally validated through a comparative analysis of precipitation (Fig. 4) and temperature (Fig. 5) within the same geographical locations. For example, the three locations of Wadi Gimal, 50 km south of Marsa Alam and 12 km north of Marsa Alam, where *Z. album* was recorded, exhibited the lowest precipitation during 2013 (Fig. 5D), 2017 (Fig. 5E), and 2021, (Fig. 5F) (0.02–0.07, 0.01–0.03, and 0.08–0.19 $mm \cdot day^{-1}$, respectively). In contrast, the same locations exhibited the highest temperature in 2013 (Fig. 5D), 2017 (Fig. 5E), and 2021 (Fig. 5F) (22.2–25.9, 21.9–26, and 22.9–26.5, respectively).

Discussion

GIS monitoring joining plants and *Eremina desertorum* occurrences

The presented findings showed that the predominant occurrences of *Eremina desertorum* were primarily observed in the vicinity of the Nile and Upper Nile Deltaic areas. Furthermore, a notable association was observed between the geographic distribution of *E. desertorum* and the presence of *Z. album* and *T. hirsuta*. This finding confirmed the influential role of these plant species in influencing the distribution patterns of *E. desertorum*, as illustrated in the geographical distribution map (Fig. 3). A significant number of land-dwelling gastropods have been observed in high quantities on various host plants, particularly in the northern region of the Delta governorates (Mahrous *et al.* 2002; EI-Deeb *et al.* 2004; Heikal 2015; Mohammed 2015; Awad and Abd El-Galil 2020) and along the North Coast belt of the Mediterranean Sea (Abdel kader et al. 2016; Ali and Ramdane 2020). Succineidae gastropods prefer wet and humid environments such as margins of lakes and rivers, and are usually found on muddy surfaces (Kerney *et al.* 1983). These gastropods are frequently observed on plants close to the banks of small rivers, as well as in the shaded areas of plant pots in nurseries and gardens, where humidity levels are higher (Ali and Ramdane 2020; Hussein *et al.* 2011).

The composition of vegetation cover plays a significant role in shaping habitat characteristics, thereby influencing population density and species abundance. It is common for live snails to inhabit various habitats such as soil surfaces, open areas, loose rocks, or vegetation. These snails often exhibit a strong attachment to these surfaces, which may be facilitated by their wide peristome (Holyoak *et al.* 2018). Ali (2017a) mentioned that the snail *E. d. desertorum* was most frequently found on certain wild desert plants: *Haloxylon salicornicum* (Amaranthaceae), *Astragalus spinosus* (Fabaceae), *T. hirsuta* (Thymelaeaceae), *Artemisia monosperma*, and *Faunaea* sp. (Asteraceae).

The findings of this study indicated that *E. desertorum* exhibited a diverse diet, encompassing multiple plant species. However, it demonstrated a notable inclination towards the consumption of the plant *Z. album,* specifically within the geographical region of Egypt. Indeed, *E. desertorum* may prefer *Z. album* over other plant species for many reasons. First, *Z. album* is a prevalent frutescent plant found in arid regions of North Africa, coinciding with the distribution of *E. desertorum* (Shaltout and Bedair 2022). Moreover, *Z. album* exhibits a wide distribution in the Northern Deltaic Mediterranean region, including locations such as Burullus Lake, National Road at Baltim, Rashid-Burullus Road, and Qalabshu in the Dakahlia governorate. Notably, approximately 90% of the localities of *E. desertorum* were found in these specific areas (Bedair *et al.* 2020). Compared to other parts of Egypt, these regions are characterized by increasing precipitation and decreasing temperature, where *E. desertorum* is preferred the most (Ali *et al.* 2016). Second, *Z. album* possesses a variety of modifications that allow it to survive in arid conditions. For instance, it has small, succulent leaves that enable it to preserve water and a deep root structure that enables it to obtain water from a significantly deep subsurface (Shaltout and Bedair 2023). These physiological features may make *Z. album* a particularly rich source of nutrients and water, which could explain the snail's preference for this plant species over others.

Furthermore, a recent study by El-Amier and Abdullah (2015) found that *Z. album* has high potential as a fodder plant for snails due to its rich nutritive value, especially its fiber, protein, and fat content, reducing sugars, carbohydrates, and energy. Similarly, lipids are an essential component of cellular

membranes and are necessary for several bodily functions (Norton 1994). It has a protein content of 19.63% and a fat content of 1.93%. In addition, it contains high amounts of nitrogen and phosphorus, followed by potassium, calcium, magnesium, and sodium. Furthermore, the examined plant microelement levels are iron (Fe) > manganese (Mn) > Zinc (Zn) > copper (Cu) > nickel (Ni) > lead (Pb) > cobalt (Co) > cadmium (Cd). Total digestible nutrition (TDN) is an estimated assessment of the food energy available to snails after digestion losses have been deduced. Weiss and Tebbe (2019) reported that the annual average TDN value was 75% DM. Indeed, the annual average TDN of *Z. album* is about 52.45% (El-Amier and Abdullah 2015).

Although there is limited research suggesting that *E. desertorum* prefers *T. hirsuta* as a food source, it is a significant shrub in the natural ecosystems of the Western Desert of Egypt, which supports a diverse arthropod fauna, including some species of snails year-round (Hegazy *et al.* 2022). A study by Shaltout (1992) indicated that *T. hirsuta* had relatively low nutrient contents of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), Fe, Mn, Cu, and Zn compared to many of the Egyptian desert plants. However, its low-growing habit may make it more accessible to ground-dwelling insects. Overall, both *Z. album* and *T. hirsuta* are desert shrubs. Their small leaves and succulent nature may provide the moist environment and water content required for *E. desertorum* growth and reproduction. In addition, their halophytic nature may possess a potential role in the diet of *E. desertorum*. Although most snails do not commonly feed on halophytes (Hasnain *et al.* 2023), *E. desertorum* may have evolved specific adaptations to cope with the high salt concentrations of these plants.

Monitoring of climate features affecting *Eremina desertorum* occurrences:

This study revealed distinct patterns of temperature changes in relation to the geographical distribution of snails and plants. This finding was confirmed by the increase in precipitation along the Mediterranean coast and Nile Delta from 2012 to 2021. Coastal areas experienced more significant temperature fluctuations than inland areas, with a noticeable negative correlation between precipitation and air temperature. The variability of gastropods can be explained by abiotic factors such as rainfall, relative humidity, and temperature (Vincent *et al.* 1982; Naggs and Raheem 2005). The presented findings were consistent with those of Kayeye *et al.* (2015), who illustrated that the densities of the recorded land snails varied seasonally. They found that the highest density occurred during the rainy season, while the lowest density was observed during the dry season. Ecological factors that may influence the distribution or variation of the recorded land snails were also determined, such as temperature, relative humidity, and rainfall. Consistent with the presented findings, Książkiewicz-Parulska and Ablett (2016) found that the interaction between habitat characteristics and weather significantly impacts snail populations and that both of these factors should be considered when interpreting monitoring results.

Several researchers have provided explanations regarding the impact of rainfall on the quality of habitats, making it suitable or unsuitable for the molluscan population and abundance (Okafor 1990; Omudu and Iyough 2005). Moreover, Myzyk (2011) reported that higher precipitation levels increased the litter moisture, resulting in a probable increase in the number of eggs laid by Vertigo angustior. According to the findings of Idowu *et al.* (2007), the fluctuations in seasonal patterns can be explained by the cyclical variations in the availability and abundance of food sources, competition among organisms, and the resumption of regular metabolic processes in individuals who have previously encountered adverse conditions. Interactions and climatic shifts within the natural environment also influence these dynamics. Ali *et al.* (2016) demonstrated that the average shell diameter of *E. desertorum* tends to increase with increasing precipitation and decreasing temperature toward the coast. Land snails are widely believed to engage in activity primarily during nocturnal hours and following rainfall events. These activities are typically observed within a temperature range of 10 to 27°C, accompanied by a relative humidity level exceeding 70%. It is further hypothesized that the growth of snails, particularly the secretion of their shells, occurs during periods with relatively moist conditions, which coincide with the snails' increased activity (Balakrishnan and Yapp 2004). The decrease in body size with decreasing precipitation and increasing temperature can be attributed to the limited feeding time in more arid regions. In contrast, snails may grow more prominent in regions where sufficient humidity allows for a more extended period of ingestion (Goodfriend 1986). During the rainy season, an increase in shell size (height) and sexual activity was observed, although snails were found in total sexual activity throughout the entire study period (Silva and Omena 2014). Precipitation controls the beginning and end of dormancy in *Helix aspersa* Muller, 1774, where periods of rainless days induce dormancy, and the return of rain indicates the end of dormancy (Iglesias *et al.* 1996).

According to Mashaly (2002), there are four primary factors: soil texture (fine fractions), moisture availability (moisture content), calcareous sedimentation (calcium carbonate), and soil fertility (organic carbon), that control how *Zygophyllum* species spread

over Egypt's Deltaic Mediterranean coastal area. Indeed, *Z. album* and *T. hirsuta* exhibit optimal growth in regions characterized by moderate temperatures and relatively high precipitation levels. Nevertheless, it has been observed that these organisms exhibit a certain degree of tolerance towards arid conditions prevalent in the desert regions of Egypt (Shaltout and Bedair 2023).

Conclusions

The results of this study revealed that the distribution of *Eremina desertorum* was primarily concentrated in the Nile and Upper Nile Deltaic regions, areas that are closely associated with the distribution of *Thymelaea hirsuta* and *Zygophyllum album*. Additionally, *Z. album* was found in three specific locations along the southern Red Sea coastline and near the Mediterranean Sea shoreline, while *T. hirsuta* was identified predominantly in northern Egypt, near the Mediterranean coast. The study further established a positive correlation between increased precipitation and the prevalence of both plant species and desert snails in these regions. This supports prior research suggesting that *E. desertorum* is associated with plant species that have a high-water content. The findings also indicated that the desert snail thrives on such plants, which may help it survive in arid environments. Therefore, when the right environmental conditions, including favorable climate and plant species, are present, the growth and reproduction of *E. desertorum* are likely to occur.

Future perspective

While spatial patterns were interpreted using previously recorded coordinates, future studies focusing on population dynamics and broader plant associations could further enhance the understanding of its habitat preferences under changing climate conditions. Moreover, future research should focus on conducting physiological and biochemical analyses of *Thymelaea hirsuta* and *Zygophyllum album* to better understand the factors that attract *Eremina desertorum* to these plants over others. Given that these plants are known to have high water content, and *E. desertorum* tends to thrive in moist environments, it is likely that this factor plays a significant role in the snail's behavior. However, it is essential to explore further the specific substances released by these plants that may influence snail habitat preference. Additionally, the impact of climate change on the physiological characteristics and adaptive responses of *E. desertorum* should be examined, as these factors could potentially alter the snail's distribution patterns. Further investigation is also necessary to provide a more detailed explanation of the association between the desert snail and these particular plant species.

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ORIGINAL ARTICLE

Natural oils as botanical acaricides: linking chemical composition, enzymatic disruption, and antioxidant capacity in the control of *Tetranychus urticae*

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Abstract

The two-spotted spider mite (*Tetranychus urticae* Koch) is a major agricultural pest, with increasing resistance to synthetic pesticides thereby, driving the search for natural alternatives. This study evaluated the acaricidal and antioxidant activities of tea tree oil (TTO, *Melaleuca alternifolia* (Maiden and Betch) Cheel), pumpkin seed oil (PSO, *Cucurbita pepo* L.), and wheat germ oil (WGO, *Triticum aestivum* L.) against *T. urticae*. Laboratory trials determined LC_{50} and LC_{90} values for adults and eggs. Biochemical effects on mites surviving TTO exposure were assessed by analyzing glutathione S-transferase (GST), acetylcholinesterase (AchE), carboxylesterase (CarE), and α-esterases activities. Antioxidant activity was evaluated via DPPH and ABTS assays. Gas chromatography-mass spectrometry (GC-MS) identified 24 compounds in TTO, with 4-terpineol (36.65%) and γ-terpinene (17.66%) as major components. TTO showed the highest acaricidal activity (LC_{50} of 0.3% for adults and 1.8% for eggs), outperforming PSO and WGO. TTO exposure significantly disrupted key enzymatic activities, impairing mite survival. Among the oils, TTO exhibited the strongest antioxidant activity. The antioxidant assays revealed that while all three oils demonstrated dose-dependent antioxidant effects, TTO was markedly more effective than PSO and WGO, although less potent than vitamin C and Trolox. Additionally, TTO exposure resulted in significant reductions in detoxification enzyme activity, particularly GST and AchE, highlighting a biochemical mechanism underlying its acaricidal action. The lipophilic properties of TTO likely enhance its penetration through the mite cuticle, increasing its efficacy. These findings support the use of plant-derived oils as eco-friendly alternatives for sustainable pest management and suggest potential for further development into natural pesticide formulations.

Keywords: AchE enzymes, antioxidants activity, GC-MS, natural oils, pesticides, spider mite, *Tetranychus urticae*

Introduction

Tetranychus urticae Koch (Acari: Tetranychidae) is one of the most economically important sucking pests (Scott *et al.* 2021). It is an extremely polyphagous herbivore that feeds on more than 1,100 plant species from at least 140 families (Rakhmanov 2024). *Tetranychus urticae* has developed resistance to more than 96 different acaricidal and insecticidal active ingredients (Adesanya *et al.* 2021), including organophosphates, pyrethroids, neonicotinoids, and even newer classes like avermectins and ketoenols. This extensive resistance is largely attributed to its short life cycle, high reproductive rate, and strong detoxification enzyme systems, such as cytochrome P450 monooxygenases, glutathione-S-transferases (GSTs), and esterases. As a result, chemical control has become increasingly ineffective in many regions. Resistance cases have been

reported in over 87 countries worldwide (Payumo *et al.* 2024), positioning *T. urticae* as one of the most resistant and difficult to manage agricultural pests globally. This highlights the urgent need to explore alternative, eco-friendly control strategies such as the use of plant derived essential oils. The "Green Revolution" significantly boosted crop productivity but came with high ecological costs, including environmental degradation, pesticide resistance, and threats to non-target species such as plants, animals, and humans. In response, there is growing interest in natural alternatives to synthetic agrochemicals (Mohammed *et al.* 2024). Among these, essential oils such as tea tree oil (TTO), pumpkin seed oil (PSO), and wheat germ oil (WGO) have been investigated for their potential in pest control (Siraj 2022).

Tea tree oil, derived from *Melaleuca alternifolia* (Family: Myrtaceae), has been traditionally used in Australia for its antimicrobial properties (Brun *et al.* 2019). Tea tree oil exhibits acaricidal, insecticidal, anti-inflammatory, antimicrobial, and antioxidant properties. The exact mechanism of its antiparasitic activity remains unclear, but it is known for its strong pest control capabilities. Pumpkin seed oil from *Cucurbita moschata* Duch (Family: Cucurbitaceae), is rich in bioactive metabolites such as carotenes, vitamin E, and phytosterols, all of which act as antioxidants (Šamec *et al.* 2022). Pumpkin seeds are byproducts widely available in various regions, including Mexico, South Asia, and the US. Wheat germ oil (WGO) from *Triticum aestivum* (Family: Poaceae), a byproduct of wheat milling, contains high levels of lipids and bioactive compounds, with different extraction methods such as solvent extraction or mechanical pressing. Pest resistance in *T. urticae* is largely driven by detoxification enzymes, including cytochrome P450-dependent monooxygenases and GST (Sharma *et al.* 2020). Essential oils offer alternative action mechanisms, potentially bypassing these detoxification pathways (Chaves *et al.* 2020).

To assess the antioxidant capacity of these oils, various spectrophotometric assays were employed, including DPPH and ABTS. Both are based on electron or hydrogen atom transfer mechanisms and quantify antioxidant activity using Trolox or vitamin C equivalents. The ABTS assay produces a blue/green $ABTS^{+}$ that antioxidants can reduce, while the DPPH assay measures the reduction of purple DPPH to a colorless form (Chaves *et al.* 2020). These methods allow for a comprehensive assessment of the antioxidant potential of natural oil samples. This study aimed to evaluate the acaricidal and antioxidant properties of TTO, PSO, and WGO against *T. urticae* using TSSM stock cultures, biochemical analyses, and oil extraction (Zieliński *et al.* 2025). TTO demonstrated the highest antimicrobial activity against *T. urticae*, followed by pumpkin seed oil and wheat germ oil. TTO also showed the highest antioxidant capacity, as measured by ABTS and DPPH assays, though it was lower than standard antioxidants like vitamin C and Trolox. These results underscore the potential of these essential oils as eco-friendly alternatives to synthetic pesticides and as natural sources of antioxidants (Mohammed *et al.* 2022b).

Buteler *et al.* (2021) studied tea tree oil to repel and affect the behavior of *Acromyrmex* spp. (leaf-cutting ants). Tea tree oil has been found to act as a repellent to certain insect pests. In laboratory bioassays, a repellent dosage response effect of tea tree oil was reported, where concentrations of 0.0001 to 0.1% tea tree oil did not impact ant behavior, but 1 and 10% concentrations repelled ants. Habashy *et al.* (2023) studied in the laboratory the potential acaricidal action of wheat germ oil (*Triticum vulgare* Vill.), clove oil and eucalyptus oil against *T. urticae*.

This study aimed to evaluate the acaricidal and antioxidant activities of three plant-derived oils–tea tree oil (*Melaleuca alternifolia*), pumpkin seed oil (*Cucurbita pepo*), and wheat germ oil (*Triticum aestivum*) against the two-spotted spider mite (*T. urticae*), a major agricultural pest. The objectives were: (1) to determine the lethal concentrations (LC_{50} and LC_{90}) of each oil on adult mites and eggs; (2) to assess the biochemical effects of tea tree oil on key detoxification and neurological enzymes in mites, including glutathione S-transferase (GST), acetylcholinesterase (AchE), carboxylesterase (CarE), and α-esterases (3) to investigate the antioxidant potential of the oils using DPPH and ABTS assays; (4) to identify the chemical constituents of tea tree oil through gas chromatography-mass spectrometry (GC-MS); and (5) to compare the efficacy of the oils as natural, eco-friendly alternatives to synthetic pesticides for sustainable pest management.

Materials and Methods

Tetranychus urticae stock cultures

Tetranychus urticae (Koch) was obtained from a susceptible colony that was maintained on *Acalypha marginata* (J.J.Sm.), a plant species belonging to the family Euphorbiaceae. The colony was reared in an incubator set at 25 ± 1°C and 65 ± 5% relative humidity (RH) in the Laboratory of Acarology, Plant Protection Research Institute, Giza, Egypt. Fresh *Acalypha* leaves were regularly replaced as needed to sustain the colony (Rott and Ponsonby 2000).

Plant collection and essential oils

The study investigated the acaricidal and antioxidant properties of volatile oils (VO) and fixed oils (FO)

extracted from three plants species: tea tree (VO), pumpkin seed (FO) and wheat germ (FO) sourced from the National Research Center Unit, Egypt, and identified by Prof. Dr. Sabry Mahfouz (Ellaithy *et al.* 2022).

Dr. Sabry Mahfouz identified the plant species used in this study, including tea tree (VO), pumpkin seed (FO), and wheat germ (FO). The VO and FO were extracted from these plants, sourced from the National Research Center Unit, Egypt, and investigated for their acaricidal and antioxidant properties (Ellaithy *et al.* 2022).

The volatile oil was extracted from the tea tree oil (TTO). After washing, drying, and crushing, the plants underwent water distillation for 4.5 h using Clevenger instruments. The oil triple samples were dehydrated with anhydrous sodium sulfate and stored for GC/MS analysis. The mean oil triple samples content was recorded (Mohammed *et al.* 2022a).

For fixed oils extraction (PSO, and WGO) (fatty acid methyl esters – FAME), 2 g of powdered fruit were extracted with 100 ml petroleum ether for 4 h using a Soxhlet apparatus. The resultant residue was weighed, and total lipid concentration was calculated. FAMEs were produced through an alkali-catalyzed reaction between fats and methanol with 2M KOH, then injected in hexane (Olubomehin *et al.* 2020).

Toxicity tests

Toxicity of tested essential oils on *Tetranychus urticae* females ovicidal test

A Petri dish was prepared with a wet cotton bed and covered with *Acalypha marginata* leaf discs (2 cm in diameter), and placed upside down. Each disc had 60 adult *T. urticae* females. Prior to the main experiment, preliminary tests were conducted to determine the appropriate concentration ranges of the three tested materials. Bioassays were then performed by applying 2 ml of each tested material, prepared in concentrations of 0.5, 1, 2, and 3% using 0.01% Tween 80 as a surfactant, onto the leaf discs with a glass atomizer. Mortality rates were assessed at 24, 72, and 120 h post-treatment.

To evaluate ovicidal activity, 10 adult female mites were placed on 2 cm *Acalypha* leaf discs set on wet cotton wool inside Petri dishes and allowed to lay eggs for 24 h under controlled conditions (27 ± 0.2°C and 55 ± 5% RH). The females were then removed and the eggs were counted. The eggs were treated with serial concentrations (0.5, 1, 2, and 4%) of the three oils. Control discs for both adults and eggs were sprayed with 2 mL of distilled water containing 0.01% Tween 80. Each concentration was tested in four replicates. Treated Petri dishes were maintained at 27 ± 2°C, 60 ± 5% RH Kumral (2010), and a 16 : 8 h light/dark cycle. Eggs were incubated at 25 ± 0.2°C and 60 ± 5% RH for seven days, after which the number of hatched and unhatched eggs was recorded.

Biochemical analysis

Adult mites were placed on *Acalypha* leaves and sprayed with LC_{50} concentration of TTO (0.3%) after 24 h. Water was applied to the control with 0.01% Tween 80. Then living mite bodies were collected from the surviving treated and non-treated mites and frozen until biochemical analysis. Carboxylesterase activity was measured according to the method described by Simpson (1964). The activity of AchE was determined using the method reported by Simpson *et al.* (1964). GST catalyzes the conjugation of reduced glutathione (GSH) with 1-chloro 2,4-dinitrobenzene (CDNB) via glutathione's -SH group. The conjugate, S-(2,4-dinitrophenyl)-L-glutathione, may be identified using the approach described by Habig *et al.* (1974). Total proteins were determined using the Bradford technique. Van Asperen's (1962) method was used to determine α- and β-esterase levels.

Chemical composition identification

GC-MS Analysis for volatile oils

The Agilent 7890B gas chromatograph and 5977A mass spectrometer with a DB-5MS column were used for the GC/MS analysis. The system used helium as a carrier gas, and the temperature program ranged from 40 to 320°C. The mass spectra were obtained through electron ionization (EI) at 70 eV, and the results were compared with the Wiley and NIST Mass Spectral Library (MSL) data for identification (Mohammed *et al.* 2022a).

Gas chromatography for fixed oils

Gas chromatography (GC) analysis of fixed oils was conducted using an Agilent 7890B system equipped with a flame ionization detector (FID) and a Zebron ZB-FAME column. Hydrogen was used as the carrier gas, with a temperature gradient set from 100 to 240°C. The injector and detector were maintained at 250 and 285°C, respectively.

In vitro antioxidant activity of three natural oils

The antioxidant activity of three oil extracts was assessed in vitro using DPPH and ABTS assays at different concentrations (1.25, 5, 10, 15, and 25 $\mu g \cdot ml^{-1}$), with ascorbic acid and Trolox serving as positive controls. $ABTS^{+}$ radical scavenging was evaluated following the method of Dinkova-Kostova *et al.* (2007), while DPPH radical scavenging followed the protocol of Mohammed *et al.* (2024). The percentage inhibition

was calculated from absorbance values (A) at 517 nm for DPPH and 734 nm for ABTS+, comparing control and test solutions side-by-side.

$$\% \text{ Inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100.$$

Statistical analysis

Mean mortality rates (± SE) of mites were calculated as a percentage of dead females. Concentration and mortality rates of adult females and eggs were calculated using Abbott's formula Abbott (1925). The LC_{50}, LC_{90}, and slope values were determined using Finney's method (Finney 1971) and analyzed with Ldp Line software as described by Bakr Dib *et al.* (2017). Biochemical study data of mite numbers was subjected to the analyses of variance test (ANOVA) with mean separation at a 5% level of significance in SAS Program version 9.1.3.

Results and Discussion

Evaluation of using essential oils and fixed oils against *Tetranychus urticae* adult females and eggs

In the current study, the acute toxicity of TTO, PSO and WGO against *T. urticae* adult females (Fig. 1) was analyzed. The four serially diluted concentrations tested were 0.5, 1, 2 and 3%, respectively. TTO had strong acaricidal activity against *T. urticae* adult females. After 24 h, TTO at 3% resulted in the greatest mean mortality rate (± SE) of 90 ± 1.92 against *T. urticae* adult females, as compared to no mortality in their corresponding control groups ($p < 0.000$). For PSO and WGO the mean mortality rate (± SE) was 86 ± 1.92 and 80 ± 2.72% at 0.3%, respectively, as compared to no mortality in their corresponding control groups ($p < 0.000$). The results showed that the LC_{50} value of TTO predicted by probit analysis for adult females at 24 h was 0.3% against *T. urticae* adult females, whereas PSO and WGO had 0.4, 0.7% LC_{50} values, respectively. After 72 h the mortality rates (± SE) were, respectively, 93.3 ± 4.71, 88.3 ± 3.19 and 86.6 ± 2.7 with TTO, PSO and WGO. After 72 h, LC_{50} values were recorded as 0.28, 0.4 and 0.5% respectively.

After 24 h the three oils recorded LC_{90} values of 4.2, 6.9 and 8.6%, respectively. Increased times of treatment increased the mortality rates. Many natural plant oils and phytochemicals have been proven to possess acaricidal action – in part due to their lipophilic nature and high vapor pressure and have no or few negative effects on non-target organisms or the environment (Anholeto *et al.* 2024). The present study revealed that all tested natural oils had acaricidal effects on *T. urticae*. The activity of the essential oils may be attributable to recognized main components. This theory is in accordance with Hussein *et al.* (2013) who reported that the most efficient natural oil was *Triticum aestivum* oil (LC_{50} = 0.995% and LC_{90} = 3.085%).

The result in Figure 2 showed that TTO was more efficient against *T. urticae* eggs at 4% with the greatest mean mortality rate (± SE) of 85 ± 1.67 followed by PSO and WGO 75 ± 4.19 and 70 ± 3.3, respectively. The LC_{50} was 1.8, 2.2 and 2.5% in TTO, PSO and WGO, respectively. The LC_{90} recorded were 5.2, 7.7 and 7.4% for TTO, PSO and WGO, respectively. Afify *et al.* (2012) reported that the LC_{50} values after 24 h for eggs were 1.17, 6.26 and 7.33% after seven days for chamomile essential oil extract, marjoram, and *Eucalyptus,* respectively. The most potent acaricidal activities were seen with chamomile essential oil extract. The results are consistent with those of Topuz *et al.* (2018) who found that the oils of the aromatic plants tested in the present reseach significantly decreased the number of laid eggs.

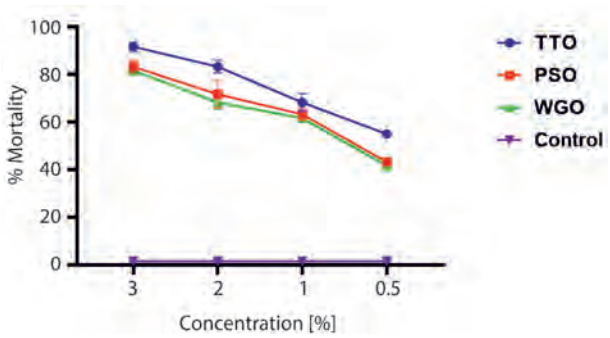

Fig. 1. Mean mortality rate (±SE) of *Tetranychus urticae* adult females treated with tea tree oil (TTO), pumpkin seed oil (PSO) and wheat germ oil (WGO) 24 h after treatment

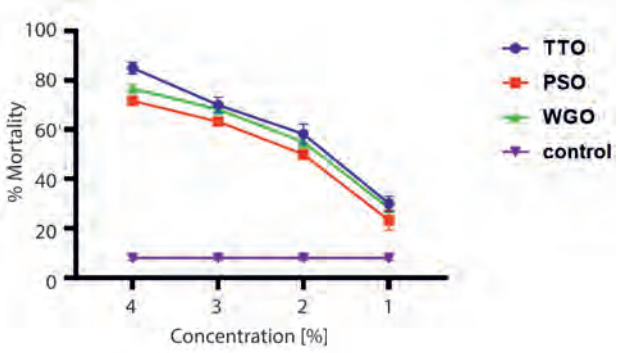

Fig. 2. Mean mortality rate (±SE) of *Tetranychus urticae* eggs treated with tea tree oil (TTO), pumpkin seed oil (PSO) and wheat germ oil (WGO)

Effect of tea tree oil on enzyme analysis in *Tetranychus urticae* adults

Changes in particular enzymes were examined in surviving mites after exposing adult females to the LC_{50} of TTO (LC_{50}, 0.3% concentration). Data in Table 1 show that a substantial reduction was found in treated mites and non-treated mites. The GST had a high significant reduction in comparison to the control that increased from 9.97 to 32.53 mmol sub.conjugated/ min/mg protein. The AchE decreased from 29.50 to 21.30 µg AchBr/min/mg protein. The CarE increased from 3.95 to 8.71 µg Meb/min/mg protein. α-esterases increased from 271 to 299.33 µg α-naphthol/ min/mg protein while there was no significant reduction in β-esterases between treated and control mites. Adly and Bakr (2016) found that no remarkable changes were observed in total proteins, alkaline phosphatase, and acid phosphatase between treated and control mites. However, treated mites that survived showed a considerable reduction in α-esterases, β-esterases and G. S-transferase on *T. urticae* with LC_{50} of menthol.

A total of 24 compounds were identified in the volatile oil of tea tree (*Melaleuca alternifolia*), representing 100% of its composition (Table 1). The main constituents were terpinen-4-ol (36.65%) and γ-terpinene (17.66%), with retention times of 8.441 and 6.461 min, respectively (Fig. 1). These results are consistent with previous international studies (Homer *et al.* 2000). Six chemotypes of *M. alternifolia* were described, each with a distinct chemical profile, including terpinen-4-ol, terpinolene, and four types rich in 1,8-cineole. The terpinen-4-ol chemotype, which contains 30–40% terpinen-4-ol, is the most frequently used in commercial tea tree oil production (Fig. 3).

A total of 11 metabolites were identified in the fixed oils extracted from WGO and PSO, collectively constituting 100% of their total composition (Table 2). Linoleic acid was the predominant constituent, representing 56.65 and 63.89% of the total oil content, with a retention time of 35.126 min (Fig. 2). These results agree with previously reported data (Shelenga *et al.* 2020) (Figure 4).

Cucurbita pepo, a member of the Cucurbitaceae family, is recognized for its nutrient-rich seeds, which are processed into PSO. Pumpkin soil oil is characterized by high levels of bioactive constituents, including essential fatty acids namely palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic acid (C18:2) as well as significant amounts of vitamin E and tocopherols (Younis *et al.* 2000). Additionally, it contains diverse phytochemicals such as tyrosol, vanillic acid, vanillin, ferulic acid, luteolin, amino acids, phytosterols,

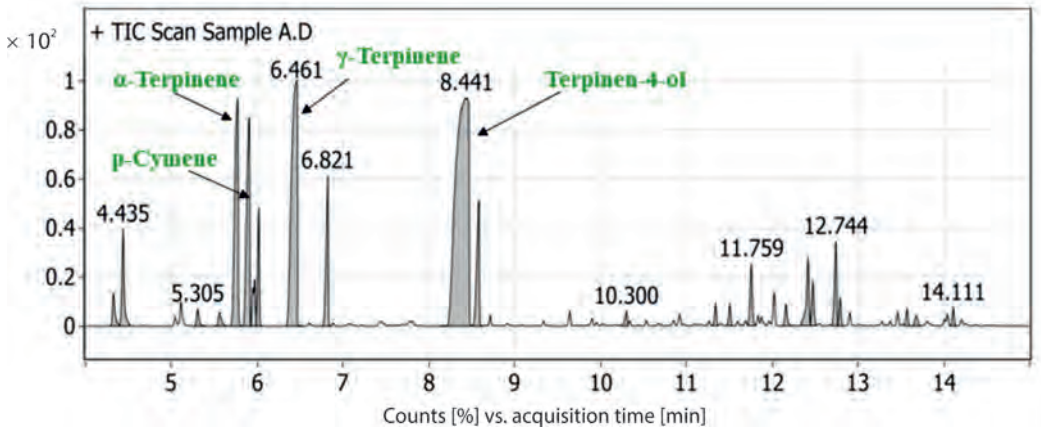

Fig. 3. TIC chromatogram of the volatile oil of *Melaleuca alternifolia*

Table 1. Glutathione S-transferase (GST), acetylcholinesterase (AchE), carboxylesterase (CarE), α-esterases, and β-esterases enzymes in the mites which survived from LC_{50} of tea tree oil (TTO) and control mites

	GST [mmol] sub. conjugated · min^{-1} · mg^{-1} protein	AchE [ug] AchBr · min^{-1} · mg^{-1} protein	CarE [ug] Meb. min^{-1} · mg^{-1} protein	α-esterases [ug] α-naphthol · min^{-1} · mg^{-1} protein	β-esterases [ug] β- naphthol · min^{-1} · mg^{-1} protein
Treated	32.53 ± 2.55 a	21.30 ± 1.61 b	8.71 ± 0.44 a	299.33 ± 7.37 a	72.43 ± 3.23 a
Control	9.97 ± 1.15 b	29.50 ± 2.0 a	3.95 ± 0.23 b	271.00 ± 8.54 b	67.53 ± 2.37 a
p-value	0.0002	0.0052	0.0001	0.0122	0.1018

Means±SD there is no noticeable change when the same letter appears in the same column are not significant difference at level 0.05

Table 2. The primary components of the volatile oil from *Melaleuca alternifolia*

Peak	Retention time	Name	Formula	Classification	Area sum [%]
1	4.326	α-Thujene	$C_{10}H_{16}$	monoterpenes	1.04
2	4.435	α-Pinene	$C_{10}H_{16}$	monoterpenes	2.75
3	5.305	β-Myrcene	$C_{10}H_{16}$	monoterpenes	0.53
4	5.562	α-Phellandrene	$C_{10}H_{16}$	monoterpenes	0.52
5	5.768	α-Terpinene	$C_{10}H_{16}$	monoterpenes	9.63
6	5.906	p-Cymene	$C_{10}H_{14}$	monoterpenes	7.87
7	5.969	ψ-Limonene	$C_{10}H_{16}$	monoterpenes	1.65
8	6.02	Eucalyptol	$C_{10}H_{18}O$	monoterpenes	2.52
9	6.461	γ-Terpinene	$C_{10}H_{16}$	monoterpenes	17.66
10	6.821	Terpinolene	$C_{10}H_{16}$	monoterpenes	3.89
11	8.441	4-Terpineol	$C_{10}H_{18}O$	monoterpenoid alcohol	36.65
12	8.584	L-α-Terpineol	$C_{10}H_{18}O$	monoterpenoid alcohol	3.85
13	10.3	1-acetyl-1,2-epoxy-2-methylcyclohexane	$C_9H_{14}O_2$	monoterpenes	0.43
14	11.342	1H-Cyclopropa[a]naphthalene	$C_{15}H_{24}$	sesquiterpenes	0.52
15	11.508	Caryophyllene	$C_{15}H_{24}$	sesquiterpenes	0.52
16	11.759	Aromandendrene	$C_{15}H_{24}$	sesquiterpenes	1.76
17	12.16	Isoledene	$C_{15}H_{24}$	sesquiterpenes	0.61
18	12.417	(+)-Ledene	$C_{15}H_{24}$	sesquiterpenes	2.25
19	12.475	β-Cyclogermacrane	$C_{15}H_{24}$	sesquiterpenes	1.17
20	12.744	δ-Cadinene	$C_{15}H_{24}$	sesquiterpenes	2.27
21	12.795	cis-Calamenene	$C_{15}H_{22}$	sesquiterpenes	0.69
22	13.573	(-)-Globulol	$C_{15}H_{26}O$	sesquiterpenes	0.43
23	13.676	Viridiflorol	$C_{15}H_{26}O$	sesquiterpenes	0.38
24	14.111	(-)-Spathulenol	$C_{15}H_{24}O$	sesquiterpenes	0.41
Total identification					100
Total monoterpenes					88.99
Total sesquiterpenes					11.01

β-carotenes, and selenium (Neamah *et al.* 2023; Singh and Kumar 2024). Pumpkin seed oil (PSO) was analyzed using GC-MS in full scan mode (m/z 60–600), and compounds were identified based on retention times and mass spectra compared to the NIST library (Mwangi *et al.* 2024) (Figure 4).

The present findings are consistent with those of Wang and Johnson (2001). US-based analyses of WGO revealed a similar fatty acid profile: linoleic acid (58.0%), oleic acid (16.4%), palmitic acid (16.4%), linolenic acid (6.4%), stearic acid (0.7%), arachidonic acid (0.2%), and eicosenoic acid (1.4%), which closely match those found in Turkish WGO (Dunford and Zhang 2003; Kan 2012). Furthermore, Zargar *et al.* (2023) identified 12 major constituents in WGO using GC-MS, accounting for 99% of its total composition. Notable components included trans-13-octadecenoic acid (99%), squalene (93%), and octadecane (94%), along with various fatty acids and terpenoids such as elaidic acid and linoleic acid, all exhibiting narrow peak widths (< 0.463), indicative of high analytical resolution. Kan (2012) summarized the fatty acid composition of solvent-extracted WGO, reporting a yield of 10.97%. The oil was comprised of seven main fatty acids, 80.1% of which were unsaturated. Linoleic acid (56.1%), palmitic acid (17.4%), and oleic acid (17.4%) were predominant, with minor fractions of linolenic acid (6.4%), eicosenoic acid (1.4%), stearic acid (0.9%), and arachidonic acid (0.2%) (Figure 4).

In this study, the antioxidant activity of three plant samples was evaluated using the DPPH assay, which showed that these plants exhibited relatively high antioxidant activity compared to other samples, though still lower than that of vitamin C and Trolox at the tested concentrations (see Fig. 1). The $ABTS^{\cdot+}$ assay measured antioxidant activity by assessing the reduction of the radical cation, represented as the percentage inhibition of absorbance at 734 nm. Figure 2 illustrates

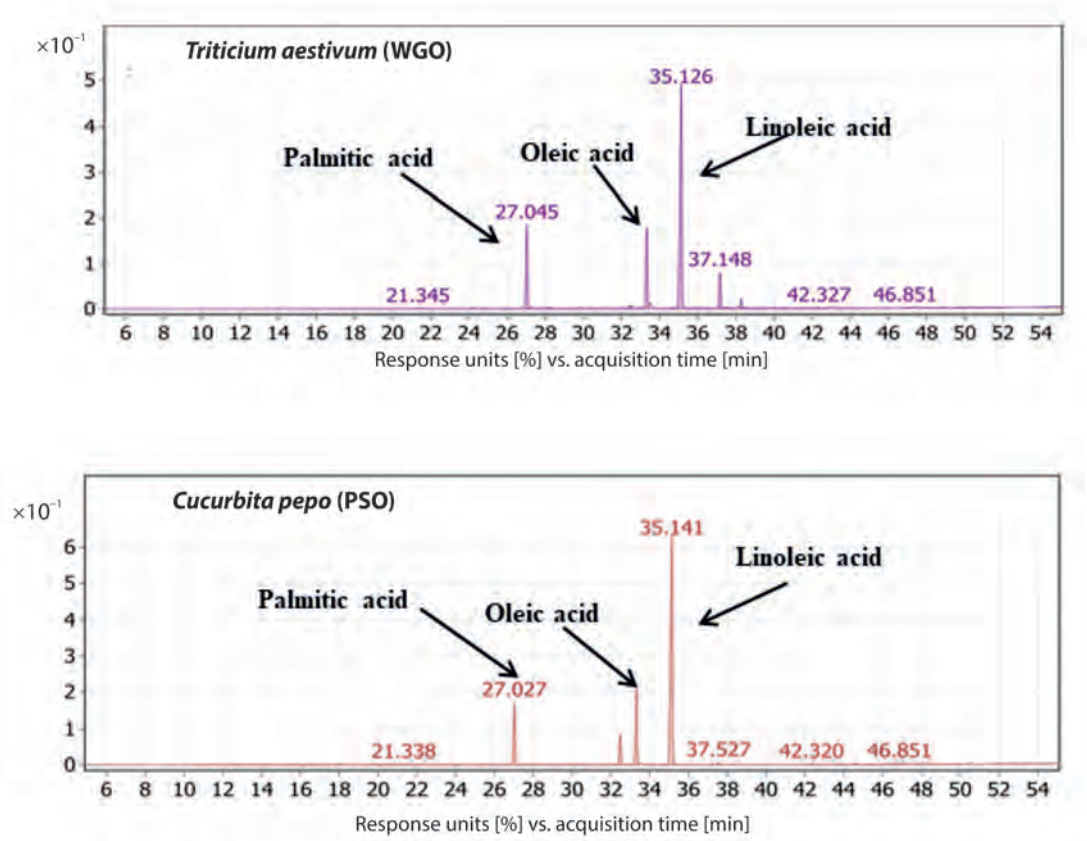

Fig. 4. Primary components of the fixed oil are *Triticum aestivum* and *Cucurbita pepo*. WGO – wheat germ oil, PSO – pumpkin seed oil

the effect of the interaction duration of specific antioxidants, such as Trolox and ascorbic acid (reference standards), on the suppression of the $ABTS^{\cdot+}$ radical cation at 734 nm. The results of the two standards were compared with those of the three plant samples (Figure 5).

The antioxidant activity was found to be concentration-dependent, with activity increasing as concentration rose. This was true for both reference standards and all three plant samples. The DPPH antioxidant activity (IC_{50} values, from highest to lowest) was as follows: vitamin C (6.231), Trolox (8.478), *M. alternifolia* (17.70), *C. pepo* (178.4), and *T. aestivum* extract (178.4). Similarly, the ABTS antioxidant activity IC50 values followed the same pattern: Trolox (7.450), vitamin C (8.847), *M. alternifolia* (9.251), *C. pepo* (57.50), and *T. aestivum* extract (121.8) (Figure 4).

The two methods used in this study to measure antioxidant activity largely confirmed each other when considering all fractions. This suggests that the extracts examined may contain similar chemical groups, and their effects can be attributed to these groups (Mohammed *et al.* 2024). In this respect, determination and characterization of such volatile oil and fatty acid profiles are useful in developing natural antioxidant substances from *M. alternifolia, C. pepo* and *T. aestivum*.

The study focused on the extraction and chemical composition analysis of volatile and fixed oils from *M. alternifolia*, *T. aestivum*, and *C. pepo*. Volatile oils were extracted through water distillation and analyzed using GC-MS, while fixed oils were analyzed with gas chromatography. Antioxidant activities of the oils were evaluated using DPPH and ABTS assays. Results indicated that *M. alternifolia* had the highest antioxidant activity among the three oils, although lower than standard antioxidants like vitamin C and Trolox.

In Table 3, the enzymatic responses of surviving mites exposed to the LC_{50} concentration (0.3%) of tea tree oil were analyzed. The results indicated a substantial reduction in the activity of GST, AchE, CarE, and α-esterases, suggesting that these enzymes play a role in detoxification and metabolism in response to the oil's active constituents. The activity of GST decreased from 9.97 to 32.53 mmol substrate conjugated/min/mg protein, while AchE activity dropped from 29.50 to 21.30 μg acetylcholine bromide/min/mg protein. The noted reductions in α-esterases and CarE reinforce the impact of tea tree oil on biochemical pathways essential for mite survival, although no significant change was observed in β-esterase activity.

The acaricidal activity of essential oils can be attributed to their lipophilic properties and high vapor

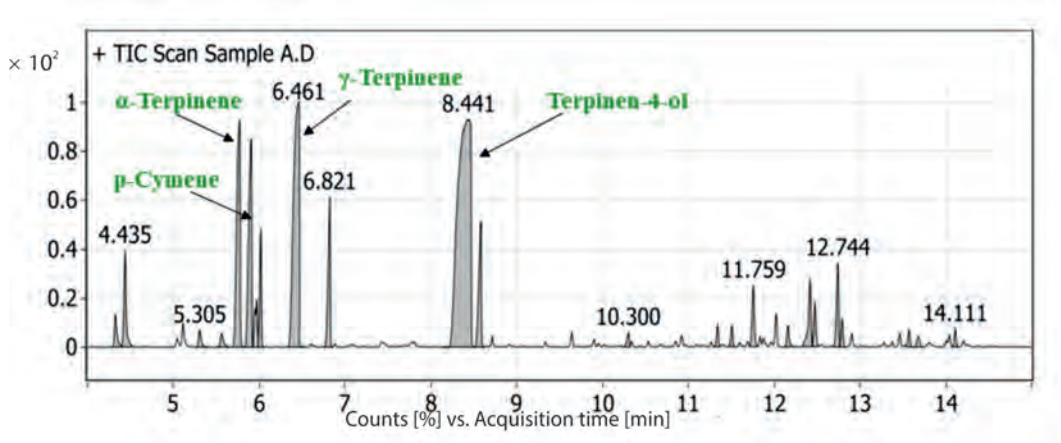

Fig. 5. *In vitro* DPPH· and ABTS·⁺ antioxidant activity of standards with three natural oil plants

pressure, which enable efficient penetration through the cuticle of arthropods, resulting in toxicity. Previous studies have highlighted the low toxicity of these compounds to non-target organisms, making them favorable for pest management. The presented findings corroborate those of Abdelgaleil *et al.* (2019), who also noted significant acaricidal effects associated with essential oils.

Regarding the efficacy against eggs of *T. urticae*, Table 3 and Figure 2 reveal that tea tree oil shows an LC_{50} value of 1.8%, outperforming pumpkin seed oil and wheat germ oil, which had LC_{50} values of 2.2 and 2.5%, respectively. The LC_{90} values corroborate this trend, with tea tree oil achieving 5.2%, compared to 7.7 and 7.4% for wheat germ and pumpkin seed oils. This is consistent with findings by Afify *et al.* (2012), who reported significant acaricidal activity in essential oils from other plants.

Tea tree oil (*M. alternifolia*) exhibited the highest acaricidal efficacy against *T. urticae*, with an LC_{50} of 0.3% after 24 h, outperforming pumpkin seed oil and wheat germ oil (0.4 and 0.7%, respectively). After three days, the LC_{50} values for all oils were 0.28, 0.4, and 0.5%, respectively. For eggs, tea tree oil had an LC_{50} of 1.8%, again surpassing the others. Enzymatic analysis revealed significant reductions in GST, AchE, and CarE activities in treated mites, indicating a disruption in detoxification processes. The primary components of tea tree oil were 4-terpineol (36.65%) and γ-terpinene (17.66%). These results suggest that essential oils can serve as effective, eco-friendly pest management alternatives. Further research is needed to optimize their application in agriculture.

TTO exerts its acaricidal effects on *T. urticae* by inhibiting key detoxification enzymes, including GST, AchE, CarE, and α-esterases, leading to metabolic

Table 3. The main constituents of the fixed oil of *Tricium aestivum* and *Cucurbita pepo*

Peak	Retention time	Name	Area sum [%]	
			Tricium aestivum	*Cucurbita pepo*
1	21.345	myristic acid	0.08	0.12
2	27.045	palmitic acid	16.9	12.51
3	28.206	palmitoleic acid	0.16	0.07
4	32.459	stearic acid	0.8	6.64
5	33.319	oleic acid	16.39	16.02
6	35.126	linoleic acid	56.65	63.89
7	37.148	linolenic acid	6.91	0.12
8	37.537	arachidic acid	0.19	0.34
9	38.244	*cis*-11-Eicosenoic acid	1.71	0.12
10	42.327	behenic acid	0.11	0.07
11	46.851	lignoceric acid	0.1	0.1

disruption (Abdelgaleil *et al.* 2019). The inhibition of AchE interferes with neurotransmission, causing paralysis and death. TTO's lipophilic nature enables efficient penetration through the mite cuticle, enhancing toxicity. The primary bioactive compounds, terpinen-4-ol and γ-terpinene, contribute to oxidative stress and membrane disruption. These mechanisms collectively impair mite survival and reproduction, supporting the use of TTO as a natural pesticide.

Conclusions

This study highlighted the potent acaricidal effect of tea tree oil (*M. alternifolia*) against *T. urticae*, with the lowest LC_{50} values among the tested oils. The observed enzymatic inhibition suggests disruption of mite detoxification pathways, likely due to key compounds such as terpinen-4-ol. These results support the use of natural oils as eco-friendly pest control agents, warranting further research to refine their agricultural application.

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A list of abbreviations

Abbreviation	Meaning
GSTs	glutathione-S-transferases
TTO	tea tree oil
PSO	pumpkin seed oil
WGO	wheat germ oil
AchE	acetylcholinesterase
CarE	carboxylesterase
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline -6-sulfonic acid)
GC-MS	Gas Chromatography-Mass Spectrometry

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ORIGINAL ARTICLE

Comparative bioefficacy of *Bacillus thuringiensis* var. *kurstaki* and neem on American white moth, *Hyphantria cunea* Drury

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Abstract

The American white moth, *Hyphantria cunea* Drury, is a polyphagous insect pest that feeds on a wide range of fruit and forest trees. In the present study, the potential of the most commonly used biological pesticide *Bacillus thuringiensis* var. *kurstaki* (Btk) and the botanical-derived insecticide neem Achook® and their combination on the mortality and physiological disruptions of *H. cunea* was investigated. The LC_{30} (1.200 and 13.350 ppm for Bt and neem, respectively), LC_{50} (3.103 and 31.753 ppm for Bt and neem, respectively), and their combinations were considered in all biochemical assays. The combination of biopesticides showed a synergistic phenomenon in all treatments at different concentrations. To explore the underlying mechanisms, we assessed the main biochemical compounds, including the activity of digestive and detoxifying enzymes of the moth larvae. Significant reductions in the activities of protease, amylase, lipase, α-glucosidase, and β-glucosidase were realized compared to the control ($p < 0.05$). The activities of detoxifying enzymes, specifically α- and β-esterases, glutathione S-transferase, and phenol oxidase, exhibited significant increases in the treated groups. Conversely, the activity of acetylcholine esterase was found to be decreased across all treatment conditions. The treatments administered resulted in a statistically significant reduction in pupal weight ($p < 0.05$). The lowest average pupal weight was recorded for the combination of *Bacillus thuringiensis* (Bt) and neem at their concentration (LC_{50}) of 98.98 mg. This research demonstrated that using Bt and neem had combined synergistic pesticidal effects that can be proposed for integrated pest management of *H. cunea*.

Keywords: biopesticides, compatibility, digestive enzymes, detoxification enzymes, pupal weight

Introduction

The fall webworm, or American white moth, *Hyphantria cunea* Drury (Lepidoptera: Arctiidae), with its origin in North America has gradually spread to central Europe and Asia (Su *et al.* 2008). As one of the key and polyphagous insect pests, *H. cunea* can cause severe damage to forests, urban trees, and crops (Kim and Kil 2012). In Iran, *H. cunea* was first recorded in August 2002 near Lasht-e-Nesha, Guilan province and it is a major pest in Northern provinces like Guilan, Mazandaran, and Ardebil (Rezaei *et al.* 2004). The populations of this pest have become widely established. They feed voraciously on a wide range of forest and fruit trees, shrubs, and herbaceous plants. More than 600 plant species have now been identified as hosts (Rezaei *et al.* 2006). The caterpillars exhibit gregarious behavior, often feeding together in large groups, which allows them to skeletonize leaves within their nests before expanding the webs, sometimes

covering entire branches (Trajković and Žikić 2023). This leads to reduced yields, aesthetic value, and plant health, with repeated damage potentially killing individual plants. The impact of the pest on fruit and berry crops can last for multiple years. The availability of food sources influences the pest's development and population dynamics, affecting its preference for certain plant species (Yanar *et al.* 2021).

Although chemical pesticides play an important role in pest control, they contaminate the environment, have adverse effects on humans and non-target organisms, and cause the emergence of pesticide-resistant populations (Kaur *et al.* 2024). As a result, there is a growing interest in shifting towards more efficient and eco-friendly alternatives, such as biopesticides, which can significantly reduce the reliance on synthetic chemicals and mitigate these negative impacts (Mawcha *et al*. 2025).

Recent trends in pest control have led to the development of many biopesticides from microorganisms (bacteria, fungi, viruses, etc.), plant and animal--derived products (pheromones, hormones, insect--specific toxins, etc.), and are being encouraged worldwide for insect pest management (Sabbour and E-Abd--El-Aziz 2010; Ayilara *et al.* 2023). The appropriate use of eco-friendly microbial pesticides can play an important role in a sustainable and scalable pest management program (Mawcha *et al*. 2025). In integrated pest management (IPM), biological control with entomopathogens is a potentially important means of reducing pest populations (Geedi and Reddy 2023). *Bacillus thuringiensis* is one of the Gram-positive spore-forming bacteria belonging to the *Bacillus cereus* group of bacilli. It produces proteinaceous crystals during sporulation which is a feature which distinguishes it from other members. Some of these crystals are toxic to the detriment of insects, nematodes, and even human cancer cells (Palma *et al.* 2014). Regarding insects, the crystals are solubilized in the midgut of insect larvae and subsequently activated by proteases. The activated toxins bind to midgut epithelial cells, forming pores and causing osmotic cell lysis, leading to insect death (Guerrero 2023). The insecticidal potential of Bt against destructive Lepidopteran pests has been documented in recent studies. For example, the susceptibility of the cutworm (*Agrotis exclamationis* L.), the velvet bean caterpillar (*Anticarsia gemmatalis* (Hübner)), the armyworm (*Spodoptera cosmioides* (Walker)), soybean looper (*Chrysodeixis includens* (Walker)), the sugarcane borer (*Diatraea saccharalis* (Fabricius)), the diamondback moth (*Plutella xylostella* (L.)), and the nettle caterpillar (*Euprosterna elaeasa* Dyar) larvae to Bt has been demonstrated (Quintero *et al.* 2020; Pinheiro and Valicente 2021; Navya *et al.* 2022; Baranek *et al.* 2023).

Several bioactive insecticidal compounds have been extracted from different parts of the neem tree (*Azadirachta indica* A. Juss.) (Sapindales: Meliaceae), of which azadirachtin is the dominant active ingredient. Multiple insecticidal effects, from repellency to molting disruption, growth reduction, development and oviposition inhibition, and high mortality of neem-based agents against several insect pests have been reported in previous studies (Valizadeh *et al.* 2013; Allahvaisi *et al.* 2021; Luo *et al.* 2023). Neem-based agents can also be used in combination with other pesticides or entomopathogens (Amizadeh *et al.* 2019; Hiremath *et al.* 2020; Bharti *et al.* 2023).

In addition to their direct lethal effects on pests, biopesticides can influence insect physiology in various ways, including altering the activity of digestive and detoxifying enzymes (Oftadeh *et al.* 2020). These agents often negatively impact insect digestive enzyme activity through direct enzyme inhibition, damage to gut epithelial cells, changes in neurotransmitter activity, and disruption of gut microbiota (Chaudhari *et al*. 2021). Detoxification enzymes, including cytochrome P_{450} enzymes, carboxylesterases, and glutathione S-transferases, play a critical role in insect adaptation and detoxification processes (Zibaee and Bandani 2010). These enzymes help insects convert lipophilic external toxins into hydrophilic compounds, effectively neutralizing the toxic effects of pesticides (Yao *et al*. 2025). These effects highlight the potential of these compounds as effective natural pest control agents.

Ensuring the compatibility of products used against target pests is a critical aspect of developing an effective pest management strategy. This involves avoiding highly toxic substances or applying them during periods when their impact on natural control agents is minimized. Studies have demonstrated that combining compatible products with entomopathogenic agents can enhance pest control efficacy, reduce the quantity of insecticides needed, lower costs, and decrease the risk of pest resistance (Sabbahi *et al*. 2022). This approach aligns with the principles of integrated pest management (IPM), emphasizing sustainable and environmentally friendly methods to effectively control pest populations.

Neem and Bt are therefore promising biopesticides for the development of integrated pest management tools. Since both Bt and neem target the digestive system of insects, their potential synergistic effects warrant investigation. Therefore, this study aimed to evaluate the effect of commercial Bt and neem products, alone and in combination, on *H. cunea*, in which, along with lethality, their effects on digestive and detoxifying enzymes were assessed. The current research was conducted to minimize or avoid the use of

chemical pesticides for the sustainable management of *H. cunea*.

Materials and Methods

Insect rearing

Eggs of *H. cunea* were collected from the forests of Rasht (37°19'N. 49°37'E.), Guilan province, Iran. The hatched larvae were reared on mulberry leaves (Kenmochi variety) under laboratory conditions: 25 ± 1°C, 75% relative humidity (RH), and 16 : 8 (light : darkness). Third-instar larvae were selected for the experiments due to their lower hair cover than older instars and high potential for damage. Body length and head capsule width were used to identify larval instars based on Dyar's law. The body length and head capsule width for third-instar larvae of this pest have been estimated to be 6–10 mm and 0.6–0.743 mm, respectively, with a Dyar's ratio of 1.631 (Mohammadi *et al.* 2010).

Insecticides

In this study, the commercial neem formulation Achook® (containing 0.03% azadirachtin), produced by Bahar Agrochem & Feeds Pvt. Ltd., Maharashtra, and marketed by Godrej Agrovet Ltd., Gujarat, India, was used. A stock suspension of *Bacillus thuringiensis* var. *kurstaki* (10^9 spore · ml^{-1}) was provided by Giah Company (Tehran, Iran), and a serial concentration was prepared using distilled water.

Bioassays

Insect bioassay was performed by the leaf disc method on the newly emerged third-instar larvae of *H. cunea* (Ebadollahi *et al.* 2013). Initial tests were conducted to select the appropriate range of concentrations. Then, five concentrations were selected based on logarithmic intervals: 3.33, 7.033, 14.67, 13.33, and 66.60 mg · l^{-1} for neem, and 0.156, 0.512, 1.25, 2.5, and 5 mg · l^{-1} for *Bt*. Each leaf (8 cm in diameter) was separately immersed in a tested concentration for 10 s, and placed in Petri dishes (150 ml) for 30 min to be dried under laboratory conditions. Control leaves were treated with distilled water. For each treatment, 10 larvae per Petri dish were used in three replicates, and mortality was documented after 24 h. The lethal concentrations (LC_{30}, LC_{50} and LC_{90}: lethal concentrations to kill 30, 50 and 90% of tested insects, respectively) were determined using Probit analysis (Finney 1971).

Determination of the synergistic effects of Bt and neem

Based on the aforementioned method, the larvae were treated separately with the mixtures of Bt + neem: $LC_{30} + LC_{30}$; $LC_{30} + LC_{50}$; $LC_{50} + LC_{30}$, respectively). After 24 h, the larval mortality was recorded and calculated based on the following formulas:

$$Em = Oa + Ob\ (1 - Oa),$$

where: *Em* – the expected mortality, *Oa* and *Ob* – the obtained mortalities of tested agents at the given concentration.

The insecticidal properties of combinations were identified as either antagonistic or synergistic by analysis using comparisons of χ^2:

$$\chi^2 = (Om - Em)^2/Em,$$

where: *Om* – the obtained mortality due to the combinations, *Em* – the expected mortality, and χ^2 – 3.84 with $\alpha = 0.05$ and $df = 1$. A value of $\chi^2 > 3.84$ and higher-than-expected mortality was considered to reveal synergistic effects, while $\chi^2 < 3.84$ demonstrated additive effects. If the observed mortality is lower than the expected mortality, it indicates an antagonistic effect between the compounds (Trisyono and Whalon 1999).

Biochemical assessments

Third-instar larvae of *H. cunea,* after treatment and in the control insects, were collected after 48 h from the onset of the experiments and then frozen at –20°C. The whole larval body was centrifuged (Thermo Fisher Scientific, Asheville, NC, USA) for 10 min at 28 600 g. The supernatant was transferred to new tubes and stored at –20°C until used. Each biochemical analysis, including digestive enzymes and detoxification enzymes, was repeated three times.

α-Amylase activity

The α-amylase activity was measured based on the method of Bernfeld (1955), using 1% soluble starch as substrate. The reaction was performed by sodium phosphate buffer (Sigma, St. Louis, MO, USA) at 35°C with 10 ml of the enzyme, 40 ml substrate, and 40 ml sodium phosphate buffer (pH 9) for 30 min. To stop the reaction, 100 ml dinitrosalicylic acid (DNS, Sigma, St. Louis, MO, USA) was added and heated in boiling water for 10 min. The absorbance was read at 540 nm using Elisa Reader (Awareness, USA). One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose as a standard curve in 30 min at 35°C.

α-Glucosidase and β-glucosidase activity

For solubilization of membrane hydrolyses (α- and β-glucosidases) in Triton X-100, membrane preparations were exposed to Triton X-100 for 20 h at 40°C, at a ratio of 10 mg of Triton X-100/mg of protein, before being centrifuged at 15,000 rpm for 30 min. No sediment was visible after centrifuging this supernatant at 22 000 g for 60 min. The activity of the enzymes remains unchanged, at –20°C, for periods of at least a month (Ferreira and Terra 1983). Incubating 50 µl of enzyme solution with 75 µl of p-nitrophenyl-α-D--glucopyranoside (pNαG, Sigma-Aldrich, St. Louis, MO, USA) (5 mM), p-nitrophenyl-β-D-glucopyranoside (pNβG, Sigma-Aldrich, St. Louis, MO, USA) (5 mM), and 125 µl of 100 mM universal buffer (pH 5.0) at 37°C for 10 min was done. The reaction was stopped by adding 2 ml of sodium carbonate (1 M) and was read at 450 nm (Ferreira and Terra 1983).

Lipase activity

The determination of lipases was conducted using the method outlined by Tsujita *et al.* (1989). In this procedure, a 10 µl homogenate solution was combined with 18 µl of p-nitrophenyl butyrate (Sigma-Aldrich, St. Louis, MO, USA) (50 mM) and 172 µl of buffer (1M, pH 7). The mixture was incubated at 37°C, and the absorbance was measured at 405 nm.

Protease activity

The general protease activity of midguts was determined using azocasein as a substrate (Elpidina *et al.* 2001). The reaction mixture was 80 µl of 2% azocasein solution (Sigma-Aldrich, St. Louis, MO, USA) in 40 mM universal buffer of specified pH and 30 µl enzyme. The reaction mixture was incubated at 37°C for 60 min. Proteolysis was stopped by the addition of 300 µl of 10% trichloroacetic acid (TCA, Sigma-Aldrich, St. Louis, MO, USA). Appropriate blanks in which TCA was added first to the substrate were prepared for each assay. Precipitation was achieved by cooling at 4°C for 120 min, and the reaction mixture was centrifuged at 16,000 rpm for 10 min. An equal volume of 1 N NaOH (Sigma-Aldrich, St. Louis, MO, USA) was added to the supernatant, and the absorbance was recorded at 440 nm.

Detoxification enzymes

To measure the activity of glutathione S-transferases according to Habing *et al.* (1974), CDNB (1-chloro-2,4--dinitrobenzene) (Sigma-Aldrich, St. Louis, MO, USA) (20 mM) was used as the substrate to measure the enzyme activity. The absorbance was recorded at 340 nm. Esterase (EST) activity was measured according to the method described by Han *et al.* (1998). For the assay, 15 µl of α-NA (10 mM) or β-NA (10 mM) was added separately to 25 µl of fast blue RR Salt (1 mM) and 15 µl of the enzyme sample.

Phenol oxidase activity assay

Ten µl of hemolymph was diluted with 90 µl of cold--sterile phosphate buffered solution and vortexed for 10 min. It was then frozen at –20°C for 48 h, after which the samples were used for phenol oxidase (PO) activity assay. The method of Cotter *et al.* (2004) was used for the assay. Centrifugation was done (5000 g at 4°C for 5 min). The supernatant (50 µl) was collected and mixed with 150 µl of L-3,4-dihydroxyphenylalanine (10 mM) (LDOPA, Sigma-Aldrich, St. Louis, MO, USA). The activity of phenol oxidase was recorded on a linear basis of phasic reactions at 490 nm. The method of Parkinson and Weaver (1999) was incorporated to determine the specific activity of PO.

Effect on pupal weight

Two-day-old third-instar larvae of *H. cunea* were fed treated leaves for 24 h. Then, until the end of the larval period (on average 10–16 days), fresh mulberry leaves were provided to the larvae every day. Each treatment had four replications (10 larvae). The weight of the formed pupae was measured with a digital scale and compared with the control group (Wang *et al.* 2009).

Statistical analysis

The probit analysis was done using the POLO-PC software (2002). In this case, significant differences between the concentrations were recorded when 95% confidence intervals (CI) did not overlap. Other data were considered for one-way analysis of variance (ANOVA) followed by Tukey's test to find significant differences between means using the SAS software (SAS Institute, Cary, Cary, NC, USA, 1997). The synergistic effect between the neem and Bt was evaluated by using χ^2.

Results

Single and combined effect of Bt and neem on *Hyphantria cunea* mortality

Significant mortality was observed in the mortality of *H. cunea* larvae treated by Bt ($F = 14.98$; $df = 3$; $p < 0.05$) and neem ($F = 17.76$; $df = 3$; $p < 0.05$). Results of the probit analysis of the lethality of Bt and neem on *H. cunea* larvae are given in Table 1, in which LC_{30}, LC_{50} and LC_{90} values were 1.20, 3.10, and 31.18 ppm for Bt and 13.33, 31.75 and 102.28 ppm for neem (Table 1). Based on low LC_{50} values and non-overlapping

Table 1. Lethal concentrations, confidence limits (95%) and regression slope lines of the effects of Bt and neem against *Hyphantria cunea* larvae after 24 h

Treatments	Slope ± SE	χ^2 ($df = 3$)	Lethal concentrations [ppm]		
			LC_{50}	LC_{30}	LC_{90}
Bt	1.280 + 253	0.515	3.103 (2.270–6.160)	1.20 (0.740–1.831)	31.18 (12.418–228.696)
Neem	1.394 ± 5.009	3.424	31.753 (16.628–216.369)	13.350 (4.943–29.284)	102.277 (45.049–1034.604)

confidence limits, *H. cunea* larvae were more susceptible to Bt than neem.

Bioassays using binary mixtures of Bt and neem demonstrated that their combination resulted in synergistic phenomena on the toxicity against *H. cunea* larvae in all the used ratios (Table 2).

Digestive enzyme inhibitory effects

Results showed that Bt and neem affected the digestive enzymatic profiles of *H. cunea* larvae in the tested concentrations. When larvae fed on leaves treated with Bt and neem, the activity of all digestive enzymes, including protease, α-amylase, lipase, α-glucosidase, and β-glucosidase**,** was decreased related to the studied concentrations (Table 3). By increasing the time from 24 h to 72 h, the activity level of enzymes gradually decreased in all treatments. The anti-nutritional effect of leaves treated with neem and Bt caused a decrease in the activity of digestive enzymes in the days after feeding. The activity of these enzymes was the lowest in the combination treatments of Bt and neem (Table 3).

Table 2. Results of binary mixture of neem and Bt against the larvae of *Hyphantria cunea*

Binary mixtures	Ratios	Larval mortality [%]				χ^2	Effect
		pure compound		binary mixtures			
		O_a	O_b	E_m	O_m		
Bt + Neem	$LC_{30} + LC_{30}$	24	18	37.68	60	13.22	synergy
Bt + Neem	$LC_{30} + LC_{50}$	24	34	49.84	76	13.73	synergy
Bt + Neem	$LC_{50} + LC_{30}$	40	18	50.8	88	27.19	synergy

O_a – observed mortality of the first compound; O_b – observed mortality of the second compound; E_m – expected mortality; O_m – observed mortality

Table 3. Effect of Bt and neem on the activity of digestive enzymes of *Hyphantria cunea* larvae

Treatments	Protease [U · mg^{-1}]	α-Amylase [U · mg^{-1}]	Lipase [U · mg^{-1}]	α-Glucosidase [U · mg^{-1}]	β-Glucosidase [mg · dl^{-1}]
Control	3.608 ± 0.0045 a	2.7195 ± 0.0045 a	3.805 ± 0.0045 a	2.0841 ± 0.005 a	2.8900 ± 0.0191 a
Neem LC_{30} 24 h	2.2541 ± 0.0065 b	2.22 ± 0.00212 b	2.969 ± 0.0015 b	2.0048 ± 0.0191 a	2.0707 ± 0.0191 b
Neem LC_{50} 24 h	2.0232 ± 0.005 c	2.150 ± 0.0021 b	2.3537 ± 0.0276 b	2.220 ± 0.0151 a	2.0075 ± 0.0525 b
Control	3.4386 ± 0.0045 a	2.5557 ± 0.0007 a	4.0018 ± 0.0341 a	1.9879 ± 0.0154a	2.7707 ± 0.0100 a
Neem LC_{30} 48 h	1.4098 ± 0.0045 b	2.1162 ± 0.0102 b	1.9744 ± 0.0012 ab	1.2895 ± 0.0141 b	1.4707 ± 0.0145 b
Neem LC_{50} 48 h	1.3819 ± 0.0082 b	2.0965 ± 0.0003 b	1.7924 ± 0.0005 b	1.2943 ± 0.0137 b	1.1703 ± 0.0261 c
Control	3.889 ± 0.0085 a	2.0785 ± 0.0003 a	3.6046 ± 0.0007 a	1.9303 ± 0.0033 a	3.0734 ± 0.0091 a
Neem LC_{30} 72 h	0.9882 ± 0.0002 b	1.273 ± 0.0056 b	1.115 ± 0.0018 b	1.4580 ± 0.0195 b	1.5707 ± 0.0198 b
Neem LC_{50} 72 h	0.748 ± 0.0285 c	1.260 ± 0.0038 b	1.0101 ± 0.0002 b	1.2451 ± 0.0143c	1.2707 ± 0.0089 c
Control	3. 231 ± 0.012 a	2.765 ± 0.0054 a	3.912 ± 0.0032 a	2.1287 ± 0.0003 a	2.7632 ± 0.0054 a
Bt LC_{30} 24 h	1.876 ± 0.0087 b	1.54 ± 0.0012 b	2.876 ± 0.0034 b	1.9854 ± 0.0098 b	2.00 ± 0.0075 b
Bt LC_{50} 24 h	1.1265 ± 0.008c	1.065 ± 0.0004 c	1.5437 ± 0.0864 c	1.054 ± 0.0043 c	1.4325 ± 0.0235 c
Control	3.1298 ± 0.0098a	2.4321 ± 0.0023 a	4.000 ± 0.0432 a	2.021 ± 0.0087 a	2.6532 ± 0.01876 a
Bt LC_{30} 48 h	1.2002 ± 0.0076 b	2.0132 ± 0.0076 b	2.9864 ± 0.0008 b	1.1432 ± 0.0008 b	1.287 ± 0.0153 b
Bt LC_{50} 48h	0.8197 ± 0.0043 c	1.9565 ± 0.0043 b	1.4321 ± 0.0054 c	0.998 ± 0.0076 c	1.0721 ± 0.0054 b

Table 3. Effect of Bt and neem on the activity of digestive enzymes of *Hyphantria cunea* larvae – continued

Treatments	Protease [U · mg^{-1}]	α-Amylase [U · mg^{-1}]	Lipase [U · mg^{-1}]	α-Glucosidase [U · mg^{-1}]	β-Glucosidase [mg · dl^{-1}]
Control	3.354 ± 0.0032 a	2.2136 ± 0.0032 a	3.654 ± 0.0023 a	2.213 ± 0.0214 a	3.123 ± 0.0032 a
Bt LC_{30} 72 h	0.7652 ± 0.0076 b	1.013 ± 0.0032 b	1.081 ± 0.0009 b	1.087 ± 0.0032 b	1.1208 ± 0.0021 b
Bt LC_{50} 72 h	0.423 ± 0.0011 c	0.7632 ± 0.0003 c	0.712 ± 0.0054 c	0.6432 ± 0.0065 c	0.5432 ± 0.0005 c
Control (Neem + Bt 72 h)	3.81037 ± 0.002 a	1.865 ± 0.0545 a	3.5577 ± 0.0026 a	2.0064 ± 0.0151 a	3.127 ± 0.0049 a
Neem (LC_{30}) + Bt (LC_{30})	0.610 ± 0.0065 b	1.189 ± 0.0038 b	0.9134 ± 0.0006 b	1.4290 ± 0.0022 b	2.6707 ± 0.0097 b
Neem (LC_{30}) + Bt (LC_{50})	0.5893 ± 0.0076 bc	0.9762 ± 0.0038 c	0.7115 ± 0.0005 bc	1.0612 ± 0.0051 bc	2.2706 ± 0.0159 bc
Neem (LC_{50}) + Bt (LC_{30})	0.548 ± 0.0088 c	0.948 ± 0.0088 c	0.5063 ± 0.0053 c	0.935 ± 0.0176c	1.6697 ± 0.0093 c

Means ± SE followed by the same letters indicate no significant difference for a given column in each treatment according to the Tukey test ($p < 0.05$)

Detoxification enzyme inhibitory effects

It was observed that treatment of the larvae by neem and Bt, alone and in combinations, caused significant changes in the activity of detoxifying esterase enzymes compared to the control group ($F = 21.43$, $df = 3$, $p < 0.0001$). The LC_{30} of neem caused a significant increase in the esterase activity regarding the β-naphthylacetate substrate. Also, an increase in the activity of general esterases (both α- and β-naphthylacetate substrates) was observed in the combination of LC_{30} values of two insecticides. Mixing LC_{50} and LC_{30} of neem and Bt, respectively, caused a significant increase in esterase activity based on the β-naphthylacetate substrate (Fig. 1).

Glutathione S-transferase activity was also increased in all treatments compared to the control ($F = 27.11$, $df = 3$, $p < 0.0001$) (Fig. 2). The highest activity of this enzyme was observed in the LC_{50} of Bt in the test with DCNB. In contrast, the highest activity of this enzyme with CDNB as substrate was observed in the combination of two insecticides at different concentrations.

Regarding acetylcholine esterase activity, unlike the other detoxifying enzymes, the enzyme activity was decreased in the larvae treated by all treatments ($F = 34.22$, $df = 3$, $p < 0.0001$). The greatest inhibition of the activity of acetylcholine esterase was detected by the combination of LC_{30} values of neem and Bt (Fig. 3).

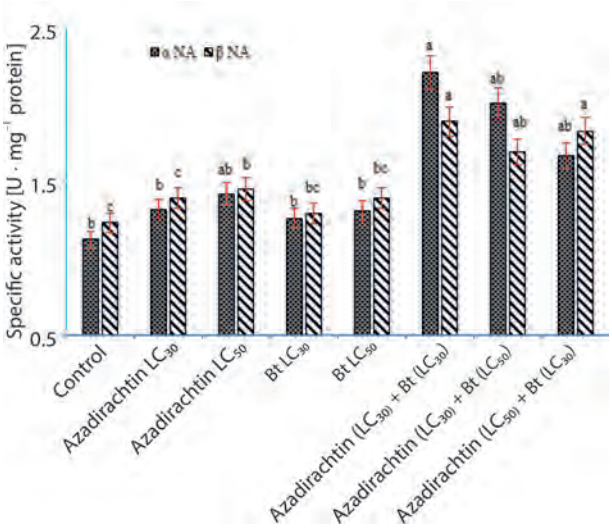

Fig. 1. Activity of general esterases in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). α NA: α-naphthylacetate; β NA: β-naphthylacetate

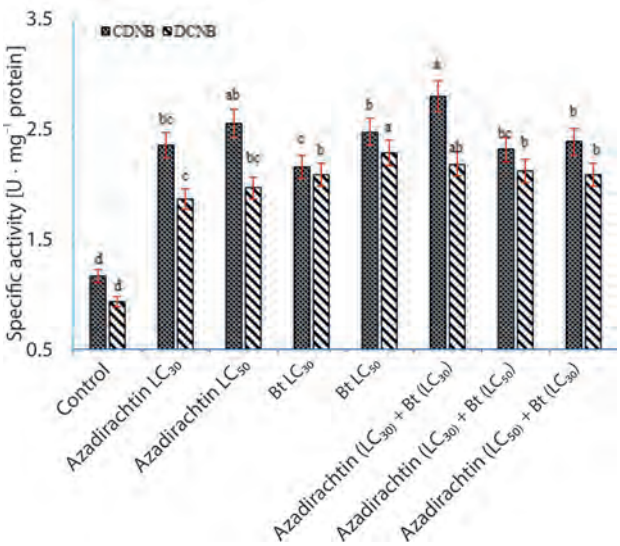

Fig. 2. Activity of glutathione *S*-transferases in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). CDNB: 1-chloro-2,4-dinitrobenzene; DCNB: 1,2-dichloro-4-nitrobenzene

Effect on phenol oxidase activity

According to Figure 4, the activity of phenol oxidase was increased in all treatments compared to the control group ($F = 21.11$, $df = 3$, $p < 0.0001$), in which the highest activity was detected in the larvae treated with neem and Bt LC_{30} – LC_{50} combinations.

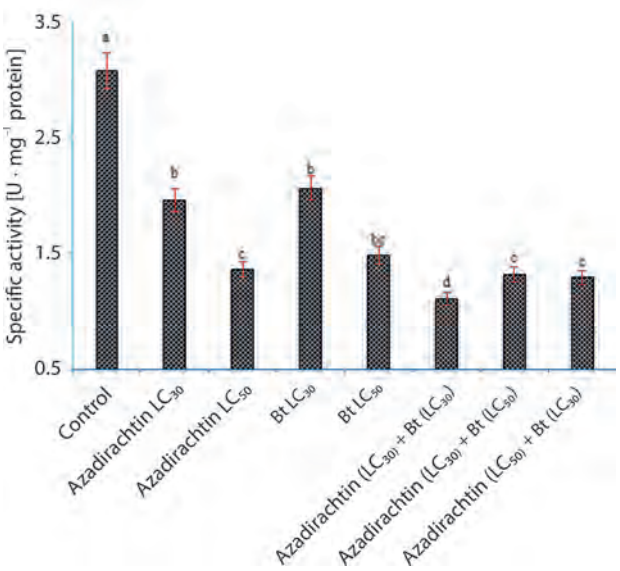

Fig. 3. Activity of acetylcholine esterase in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$)

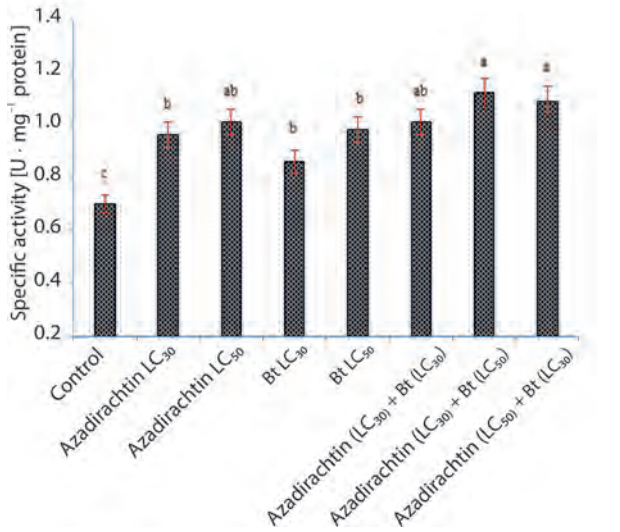

Fig. 4. Activity of phenol oxidase in the *Hyphantria cunea* larvae treated with Bt and neem. Different letters indicate significant differences between treatments according to Tukey test ($p < 0.05$)

Effect on pupal weight

According to Table 4, the weight of *H. cunea* pupae emerged from larvae affected by all separate and combined treatments of neem and Bt was significantly decreased compared to the control group ($F = 68.67$, $df = 3$, $p < 0.0001$). The neem LC_{50} treatment and the combination of neem LC_{50} + Bt LC_{30} resulted in the most significant reduction in pupal weight compared to all other treatments. Indeed, neem, present in all treatments, had a high inhibitory effect on the feeding of larvae, and therefore, incomplete pupae with low weights emerged from weak larvae.

Table 4. Pupal weight (mg ± SE) emerged from *Hyphantria cunea* larvae affected by neem and Bt (mean of both sexes)

Treatments	Pupa weight [mg]
Control	207.08 ±0.87 a
Neem (LC_{30})	127.23 ±3.37 bc
Neem (LC_{50})	110.31 ±5.09 cd
Bt (LC_{30})	172.65 ±3.12 b
Bt (LC_{50})	130.72 ±3.33 bc
Neem (LC_{30}) + Bt (LC_{30})	130.54 ±11.07 bc
Neem (LC_{30}) + Bt (LC_{50})	116.08 ±5.32 c
Neem (LC_{50}) + Bt (LC_{30})	98.98 ±0.07 d

Means ± SE followed by the same letters indicate no significant difference for a given column in each treatment according to the Tukey test ($p < 0.05$)

Discussion

Biorational pesticides are environmentally friendly as they possess biodegradable and low-risk features compared to classic synthetic pesticides (Horowitz *et al.* 2009). Furthermore, due to minimal residual effects, predators, parasitoids, and pollinators are the least affected, and accordingly, they are compatible with IPM programs (Murugesan *et al.* 2023). Biopesticides, with their multiple modes of action and diverse insecticidal targets, provide significant advantages in managing pest resistance, driving growing interest among researchers (Birch *et al.* 2011). In the present study, the high mortality rate, the inhibition of digestive enzymes and acetylcholine esterase activity, and the reduction in emerged pupae weight were revealed on *H. cunea* larvae treated with Bt and neem, either individually or in combination.

According to the present findings, the combination of different concentrations (LC_{30} and LC_{50} values) of Bt and neem resulted in a synergistic phenomenon in the mortality of *H. cunea* larvae. Similarly, Singh *et al.* (2007) presented a positive interaction between Bt (Delfin® WG™ (Certis USA, LLC) and neem (500 ppm azadirachtin (AI)) on the larval mortality of the cotton bollworm *Helicoverpa armigera* Hübner. Although they concluded that neem facilitated the action of Bt, the combined action was not synergistic. Abedi *et al.* (2014) showed that a combination of neem and Bt can be more effective on *H. armigera* than using them alone to control this pest. In another study, positive interactions of Bt and neem on larvae of the Indian meal moth *Plodia interpunctella* Hübner were reported (Nouri-Ganbalani *et al.* 2016). The results of these studies emphasize the present findings about enhancing the insecticidal potential of Bt and neem by their combination. However, differences in lethal

concentrations and synergistic or additive phenomena can be justified by different studied insect pests.

The activity of enzymes reflects the absorption, digestion, and positive transport of nutrients through the midgut. The Bt has been reported to cause damage to the epithelial cells of the midgut through crystalline parasporal bodies, which release the active toxin after digestion by serine proteases under alkaline conditions in the intestinal juice; the damage to the midgut causes a decrease in enzyme activities (Mathavan *et al.* 1989; Miao 2002). Diet treatment with botanical insecticides can also affect several enzyme activities in larvae of *H. cunea* by interfering with the production of certain types of proteins (Senthil-Nathan *et al.* 2006). Therefore, it may be expected that such damage to the midgut would cause a serious reduction in the activity of digestive enzymes.

The study revealed that Bt and neem treatments significantly reduced protease activity, suggesting disruptions in both digestive enzyme production and midgut epithelial integrity. Proteases play a critical role in insect digestive processes and metabolic functions. The observed decline in protease activity likely impairs nutrient absorption and disrupts cellular homeostasis, highlighting the profound physiological impact of these treatments on target insects (Klowden 2007). Lipases, vital for growth, reproduction, and pathogen defense, are reduced post-treatment with Bt and neem, indicating a blockage in the conversion of phytosterols to lipase building blocks (Klowden 2007). There is a reduction in the activity level of α- and β-glucosidases. This may be due to a drop in consumption rates and leveling off or a decline in food conversion efficiencies (Zibaee *et al.* 2009). The observed reduction in α-amylase in this study may result from damage to the midgut epithelium, which is responsible for enzyme production (Senthil-Nathan *et al.* 2006). Bt and neem have been shown to significantly reduce the activity of digestive enzymes in various insect species, thereby impairing their ability to process nutrients effectively (Senthil-Nathan *et al.* 2009). Studies have demonstrated that these botanical products can decrease the activity of proteases, amylases, and lipases in other insects such as southern armyworm *Spodoptera eridania* Stoll and the gypsy moth *Lymantria dispar* (L.) (Shannag *et al.* 2015; Zou *et al.* 2019). This reduction in enzyme activity not only impairs nutrient absorption but also leads to antifeedant effects, reduced food consumption, and slower larval growth (Mordue(Luntz) and Nisbet 2000). These effects, observed in previous studies on applying botanical compounds, highlight the disruption of digestive enzyme synthesis in response to substrate presence in the midgut (Oftadeh *et al.* 2020, 2021).

Increases in glutathione S-transferase (GST) activity and decreases in the AChE activity as important detoxifying enzymes by the combination of neem and Bt were observed in the present study. Potentially, this indicates that while GST increased to detoxify the biopesticides, an inhibitory effect was observed with AChE. Similar results were observed in the application of the essential oil of Ajwain (*Carum copticum*, L.) on *Chilo suppressalis* Walker (Basij *et al.* 2023). War *et al.* (2014) observed that a combined treatment of neem oil formulation and endosulfan reduced esterase activity but increased glutathione-S-transferase activity in *H. armigera* larvae. The application of neem and Bt has been shown to increase the activity of some detoxification enzymes in insects as part of their defense mechanisms. For instance, Bt treatment in two commercial formulations in *Spodoptera litura* (F.) has been found to elevate phenol oxidase activity after 48 h, which plays a crucial role in melanization and pathogen defense (Kamel *et al.* 2010). Similarly, exposure to neem in the palmetto weevil *Rhynchophorus cruentatus* F. led to increased levels of detoxification enzymes such as glutathione S-transferase, and superoxide dismutase, aiding in the breakdown of toxic compounds (Gabr *et al.* 2022). In this study, despite these increases, both neem and Bt reduced the activity of acetylcholinesterase, an enzyme critical for nerve function, thereby impairing the insect's ability to develop resistance, particularly when these compounds were used in combination.

Bt and neem induced significant insecticidal effects on third-instar larvae of *H. cunea*. Higher mortality was observed when the larvae fed on leaves treated with Bt and neem combinations than larvae that fed only on a diet containing one of these insecticides. Furthermore, Bt and neem significantly reduced the activity of digestive enzymes and pupae weight along with an increase in the activity of acetylcholine esterase. Exposure to a combination of Bt and neem led to a greater effect of these parameters. It can be suggested that the use of neem as a synergist to a Bt with multiple modes of action may result in a more effective management strategy against *H. cunea*. However, to apply the research results, future research should focus on field-level studies and investigate the combination of weather and temperature on these natural products to find the suitable time for using or protecting these compounds. Such studies could provide new insights into the use of botanical agents as additives to Bt, which may play a more prominent role in pest control programs in the future.

Conclusions

Hyphantria cunea is a relevant insect pest causing significant damages to fruit, ornamental plants, forest

trees and other crops. The extensive use of synthetic insecticides to control this pest raises environmental and health concerns, highlighting the need for alternative methods. Investigating bio-pesticides is critical to support IPM programs, as they offer a safer and more sustainable approach to pest control. This study found that the combination of neem and Bt was effective in suppressing *H. cunea* due to their synergistic effect. The potential benefits of this synergistic effect on IPM strategies are substantial: the combination of neem and Bt has been shown to be effective against *H. cunea* larvae, particularly in reducing digestive enzyme activity and pupal weight. Treated larvae also showed a significant decrease in detoxifying enzyme activity. Conducting such analyses will provide valuable insights to optimize pest control while prioritizing safety and efficacy.

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ORIGINAL ARTICLE

Enhancing drought tolerance in *Lippia graveolens* through ethyl methanesulfonate (EMS)-induced mutagenesis

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Abstract

Mexican oregano (*Lippia graveolens*), belonging to the Verbenaceae family, is an aromatic and perennial herb that produces an essential oil rich in the monoterpenes thymol and carvacrol, widely utilized in various industries. Endemic to Mexico, it predominantly thrives in arid and semi-arid regions, typically displaying notable drought tolerance. However, previous studies reveal that irrigation frequency significantly influences biomass production, prompting the need for further improvement in drought tolerance in this species, especially when considering future climate change scenarios. This study employed chemical mutagenesis with ethyl methanesulfonate (EMS) to create new genetic variants through induced mutations. Seeds of *L. graveolens* underwent EMS treatment at varying concentrations (0.1 and 0.2%) and exposure times (1, 3 and 6 hours), and then aseptically germinated on MS medium. Nodal segments from resulting seedlings were used as explants for multiple shoot proliferation using 50 g · l^{-1} of polyethylene glycol (PEG) as a selective agent for drought tolerance, where non-mutagenized plants displayed severely inhibited development and necrosis. Twenty-five putative mutants tolerant to osmotic stress were recovered, and some of them showed evident morphological alterations and significant changes in the content of phenols and flavonoids, compounds associated with responses to stress. These results highlight the effectiveness of chemical mutagenesis as a strategy for genetically enhancing drought tolerance in Mexican oregano.

Keywords: drought stress, *in vitro* culture, Mexican oregano, phytochemistry, polyethylene glycol

Introduction

Lippia graveolens, commonly known as Mexican oregano, is an aromatic perennial shrub that belongs to the Verbenaceae family. It is considered to be a non-timber forest species, with organoleptic and medicinal properties that make it highly attractive for the pharmaceutical, cosmetic and food industries, being one of the most commercially relevant species within this family. The plant is endemic to Mexico and it is mainly located in arid and semi-arid regions in the central part of the country. It has oval-shaped serrated leaves with pointed tips, a hairy woody stem and small white flowers. The plant is a rich source of bioactive compounds with several properties (e.g., antioxidant, antimicrobial, and anti-inflammatory). Its commercial value stems largely from its essential oil, characterized by a high content of the phenolic terpenes thymol and carvacrol (Muñoz-Miranda *et al*. 2019; Bautista-Hernández *et al.* 2021; Bautista-Hernández *et al*. 2023). One of the most common applications is the use of its essential oil as a conservative for packaged food and as a broad-spectrum antimicrobial due to its remarkable inhibitory effect on the growth of bacteria (Hernández-Hernández 2019; Reyes-Jurado *et al*. 2020) and fungi such as *Candida albicans* (Herrera-Rodriguez *et al.* 2019).

Mexican oregano is adapted to grow under low water availability, which makes it a suitable alternative for agricultural production in arid areas of Mexico. Although oregano plants do not require frequent watering in the early stages of growth, it has been shown that in latter stages there is a linear relationship between the amount of water applied during irrigation and the overall plant biomass production (Llamas-Torres *et al.* 2022). This behavior suggests that water availability significantly influences the harvest yields of oregano. Therefore, projections of future drought affecting the distribution area of this species, as per climate change scenarios, are anticipated to have a considerable adverse impact on oregano production (Balting *et al.* 2021). Climate change directly impacts the agricultural sector by increasing various types of abiotic stress, such as drought and salinity, leading to reductions in crop yields, which can exceed 50% worldwide. The closure of stomata, as a mechanism to protect against water loss, limits the CO_2 concentration, inhibiting the productivity of the photosynthetic process. Lowering of CO_2 levels also promotes the formation of reactive oxygen species (ROS), which, in turn, cause the oxidation of important molecules such as proteins and lipids. Besides its direct effect on plant physiology, abiotic stress also severely affects agricultural productivity by causing a reduction in genetic diversity and increasing the risk of germplasm loss (Muscolo *et al.* 2014; Kopecká *et al.* 2023).

Various biotechnology approaches have been employed to enhance abiotic stress tolerance in plants. One of the most frequently used methods, mutation breeding, relies on applying physical or chemical mutagens to plant material to artificially increase the number of mutations and expand the genetic variability of plant species of agronomic interest. This approach has contributed to the creation of plant varieties with enhanced phenotypic characteristics, including environmental stress tolerance (Channaoui *et al.* 2019b; Mullins *et al.* 2021). In the case of plants containing bioactive compounds, mutagenesis allows for the generation of varieties with a higher content of these metabolites (Shah *et al.* 2020). Ethyl Methanesulphonate (EMS) stands out as one of the chemical mutagens commonly used in mutation breeding, typically inducing random point mutations in the plant genome. The dosage and duration of EMS application are crucial factors influencing mutagenesis efficiency (Channaoui *et al*. 2019a; Olaolorun *et al.* 2019). Noteworthily, EMS mutagenesis has also been effective for the development of novel stress-resistant microbial agents that exhibit plant-beneficial attributes, offering potential solutions for agricultural challenges posed by climate change (Wongwanich *et al.* 2017).

After mutagenesis, to select phenotypes of interest in the laboratory, efforts are made to recreate the conditions to which the plants will be exposed. The goal is to identify those individuals that exhibit better development or fewer impairments in such an environment. In order to mimic drought stress under *in vitro* conditions, a variety of compounds called osmolytes have been used as selective agents when added to the culture medium. Two of the most commonly used osmolytes are mannitol and polyethylene glycol (PEG), which act by lowering water potential, making it more difficult for plants to absorb water from the medium. Using this procedure, drought-tolerant mutants of crops such as alfalfa, sugarcane, rapeseed and radish have been successfully obtained (Khalil *et al.* 2018; Tiryaki *et al.* 2022; Chen *et al.* 2023; Gonzalez *et al*. 2024).

Previous attempts have been made to apply EMS-induced mutagenesis in order to increase genetic diversity in Mexican oregano. In that context, nodal segments were used as plant material and the effect of the mutagen on the *in vitro* regeneration rate was evaluated. The genetic variability of the plants obtained was assessed using molecular markers (Muñoz-Miranda *et al.* 2019). The main goal of the present study was to obtain mutant plants of *Lippia graveolens* through the exposure of seeds to EMS and the selection of lines that showed improved performance under drought stress induced by either mannitol or PEG.

Materials and Methods

Plant material

Seeds were obtained from *L. graveolens* plants collected from wild populations in the municipality of Colotlán, Jalisco, at the following geographic coordinates (via GPS): 22°01'43.12" N 103°15'01.9" W and 22°01'44.2" N 103°14'41.0" W. Seeds from two plants were sampled at each location and pooled to obtain a single bulk used for all experiments. The collection site is located at 1,720 meters above sea level.

In vitro germination

For disinfection, seeds placed in Eppendorf tubes were first soaked in 70% ethanol for 1 min and then rinsed three times with sterile distilled water (1 min each). Subsequently, washed seeds were immersed for 3 min in 30% commercial bleach (1.68% available sodium hypochlorite) and rinsed three times with sterile distilled water for 1 min. Half-strength MS medium (Murashige and Skoog 1962) was used for the germination of disinfected seeds. This medium was supplemented with 1% sucrose and 0.25% Phytagel™, and the pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. Seeds placed on MS medium were incubated at 24°C

with a photoperiod of 16/8 h light/dark under white fluorescent lamps with an intensity of 32 μmol · m^{-2} · s^{-1} (Muñoz-Miranda *et al.* 2019).

Formulation of culture media for selection of mutants

Mannitol and PEG 6000 were added to the culture medium to simulate drought stress. To determine the minimal concentration at which these osmolytes impair plant growth, culture medium was prepared as full-strength MS with 3% sucrose and 0.25% Phytagel™, and the pH was adjusted to 5.8 before autoclaving. This medium was supplemented with either mannitol (50, 100 and 150 mM) or PEG (10, 25 and 50 g · l^{-1}). Nodal segments obtained from *in vitro* germinated seedlings were placed on each medium. MS medium without any of the osmolytes was used as a control. Each treatment was comprised of three explants per flask with 10 replicates. After 30 days, morphological characteristics of the shoots produced from the axillary buds were evaluated. Features such as color of the tissue, presence of hyperhydric malformations (vitrification), necrosis, and overall survival were recorded.

Chemical mutagenesis

After disinfection, selected seeds were exposed to a sterile aqueous solution of EMS at two concentrations (0.1 and 0.2%) with three exposure times (1, 3 and 6 hours). EMS concentrations and exposure times were established based on those commonly used for seeds and previous experience with the mutagenesis of vegetative tissues in *L. graveolens* (Muñoz-Miranda *et al.* 2019; Chen *et al.* 2023). One-hundred seeds were used for each treatment. A control without EMS was also included. After the treatments, the seeds were rinsed seven times with sterile distilled water (1 min each) to remove excess EMS. Mutagenized seeds were then sown on MS medium prepared as previously described for seed germination.

Selection and micropropagation of putative mutant plants

The micropropagation of putative mutant *L. graveolens* plants was carried out according to the procedure described by Castellanos-Hernández *et al.* (2013). M1 plants derived from EMS-mutagenized seeds were used as the source of explants. Three nodal segments from these plants were placed on selective MS medium containing the appropriate concentration of either mannitol or PEG for selection of mutants as determined previously, and supplemented with 2 mg · l^{-1} of benzyladenine (BA). After 100 days, plants that successfully coped with osmotic stress and did not exhibit severe growth impairment were transferred to MS medium without the addition of the osmolyte for regeneration. Following regeneration, plants showing better performance were selected for a second cycle of micropropagation.

Measurement of morphological traits

Two months into the second cycle of micropropagation, the morphological characteristics of the plants such as the number of activated buds, plant height (only aerial part), and the number of leaves were evaluated.

Phytochemical analysis

Preparation of the crude extract

One plant from each putative mutant genotype was selected for phytochemical analysis. For each plant, three replicates, consisting of two leaves, were prepared. The weight of the leaves was recorded, and methanolic extracts were obtained by placing the leaves in Eppendorf tubes and adding 1.5 ml of methanol. The tubes were left for 24 hr with continuous agitation. The liquid was recovered in a new tube and this crude methanolic extract was used for further determinations.

Determination of total flavonoid content (TFC)

The total flavonoid content was quantitatively determined as described by Pedro *et al.* (2023). A standard solution of quercetin in methanol was used to plot a calibration curve in the concentration range of 10–100 μg · ml^{-1}. The determination of TFC was performed by mixing 500 μl of crude methanolic extract with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. This mixture was incubated for 30 min at room temperature, and then absorbance was measured at 415 nm using a spectrophotometer. Results were reported as mg of quercetin equivalents per 1 g of fresh weight (mg QE · gfw^{-1}).

Determination of total phenolic content (TPC)

The total phenolic content of the extracts was estimated using the Folin-Ciocalteu method as described by Waligóra *et al.* (2023) with some changes. First, the Folin-Ciocalteu reagent was diluted to a ratio of 1 : 10 with distilled water; then, 1 ml of this diluted reagent was mixed with 200 μl of the crude methanolic extract and incubated for 1 min at room temperature.

Subsequently, 0.8 ml of 7.5% sodium carbonate was added, and the mixture was incubated for 1 hr at room temperature. Following the same procedure, a standard solution of gallic acid in methanol was used to construct a calibration curve in the concentration range of 25–200 μg · ml^{-1}. The absorbance was measured at 765 nm using a spectrophotometer and results were expressed as mg of gallic acid equivalents per 1 g of fresh weight (mg GAE · gfw^{-1}).

Statistical analysis

Values were expressed as means ± standard deviation (SD). Data were analyzed using one-way analysis of variance ANOVA and Post-hoc analysis was conducted using Tukey's pairwise comparison. A multiple range test was carried out to determine whether significant differences occurred between individual treatments at a significance level of $p < 0.05$. The software Statgraphics Centurion XVI v.16.103 was used for all analyses.

Results

Selective medium

Lippia graveolens seeds were not directly sown on MS medium containing either mannitol or PEG since germination was severely inhibited by both osmolytes (data not shown). Therefore, seeds were first germinated on MS without these compounds, and then nodal explants from the resulting seedlings were taken for micropropagation on medium supplemented with the osmolytes. The explants exposed to mannitol did not show evident alterations in their development at any of the concentrations used. For this reason, this osmolyte was not subsequently used as a selective agent for the identification of putative mutants tolerant to drought stress. On the other hand, PEG did affect the growth of the plants when added to the culture medium, with this effect being more pronounced at 50 g · l^{-1}. At this concentration, plants showed hyperhydricity (formerly known as vitrification), chlorosis, and finally necrosis by the end of the incubation period (Fig. 1a). Hence, MS medium supplemented with PEG 50 g · l^{-1} was used as a selective medium for the identification of mutant plants showing enhanced tolerance to drought stress.

Chemical mutagenesis

Exposure to EMS affected the germination rate of the seeds at all doses and times tested, although there was a general trend of decreased germination rates with increased dose and exposure time (Table 1). Seedlings that germinated and grew after the mutagenesis treatment were used as a source of explants for micropropagation on selective medium containing PEG. Plants developed from explants, showing no evident growth impairment, were considered to be putative mutants tolerant to drought stress. Representative images of the putative mutants are shown in Fig. 1. A total of 25 of these tolerant plants were obtained (Table 1) and transferred to MS medium without PEG for micropropagation and further morphological and phytochemical analyses. Putative mutant plants were labeled using codes LGM01 to LGM25.

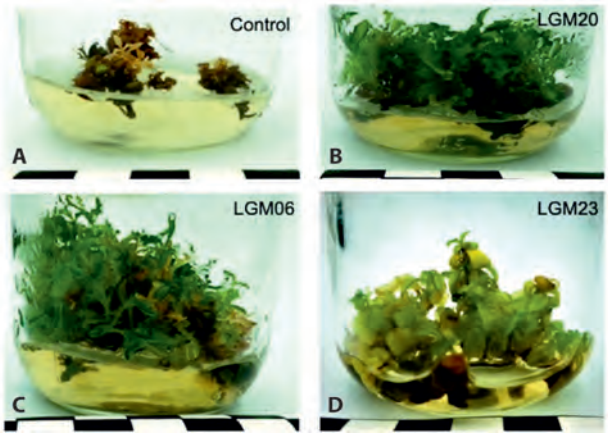

Fig. 1. Selection of putative EMS-induced drought-tolerant *L. graveolens* mutants. Development of nodal segments from A – non-mutagenized oregano plants, and three putative mutants: B – LGM20, C – LGM06 and D – LGM23, after 100 days on MS medium supplemented with PEG 50 g · l^{-1} as a selective agent

Table 1. Effect of EMS treatments on the germination rate of *Lippia graveolens* seeds

	EMS Concentration						
	Control	0.1%			0.2%		
	Exposure time [h]						
	0	1	3	6	1	3	6
Germination rate	65%	26%	11%	4%	18%	6%	5%
Number of putative mutant plants	–	9	4	1	7	3	1

Table 2. Morphological characteristics of selected *Lippia graveolens* mutant plants

	Mutant plants						
	control	LGM06	LGM11	LGM14	LGM20	LGM23	LGM25
Number of leaves	15 ± 1.0 b	14 ± 1.0 b	11 ± 2.0 c	20 ± 3.0 a	9.0 ± 0.5 c	11 ± 1.0 c	20 ± 3.0 a
Number of axillary buds	18 ± 1.0 a	6.0 ± 0.2 b	3.5 ± 1.0 b	20 ± 2.0 a	4.0 ± 1.0 b	4.5 ± 0.5 b	20 ± 2.0 a
Plant height (cm)	13.6 ± 1.0 c	21.3 ± 1.5 a	18.1 ± 1.0 b	4.0 ± 1.5 d	24.3 ± 2.0 a	3.5 ± 0.5 d	4.0 ± 1.5 d

Values are expressed as the mean ± standard deviation (SD). Letters next to the values represent homogeneous groups; within each row, different letters indicate significant difference at $p < 0.05$ using ANOVA and Tukey's HSD test

Morphological characteristics

Putative mutant plants showing optimal development after two cycles of micropropagation on MS medium supplemented with benzyladenine were selected for analysis of their morphological traits. Some of these plants displayed noticeable differences in height, number of leaves, and the proliferation of axillary buds compared to non-mutagenized plants, as shown in Table 2. Even though those characteristics were not measured, in several of the potential mutants obtained, noticeable alterations were observed in the color, size, and shape of the leaves, as well as the abundance of trichomes. Representative images of the putative mutants, alongside the control plants, are shown in Figure 2.

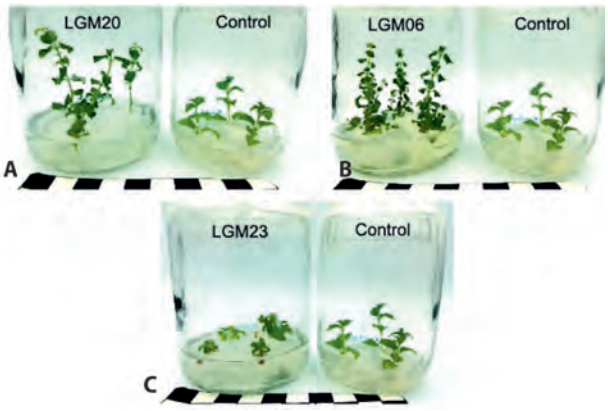

Fig. 2. Morphological variations observed in EMS-induced *Lippia graveolens* mutants during micropropagation. Development of nodal segments from PEG-resistant plants after 1 month on PEG-free medium for micropropagation: A – LGM20, B – LGM06, C – LGM23. In all panels, the flask on the right contains non-mutagenized control plants

Phytochemical analysis

The total flavonoid content (TFC) in the *Lippia graveolens* mutants varied significantly across the different plant samples evaluated (Fig. 3). Mutants LGM14, LGM20, LGM23, and LGM25 exhibited notable increases in TFC compared to the control plants

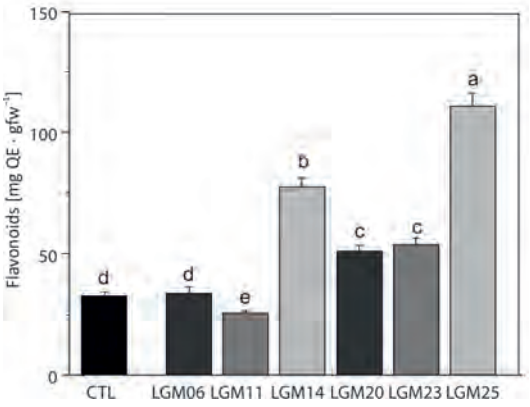

Fig. 3. Quantitative determination of total flavonoids content (TFC) of *L. graveolens* mutants. Means of the TFC for three replicates and standard deviation are shown. Different letters above the bars indicate significant differences ($p < 0.05$). Control (CTL) corresponds to non-mutagenized oregano plants

(CTL). In particular, mutant LGM25 showed the highest flavonoid content, reaching approximately 130 mg QE · gfw^{-1}, which was significantly higher than all other samples ($p < 0.05$). LGM14 and LGM23 also demonstrated a significant increase in TFC relative to the control, with values around 80 and 70 mg QE · gfw^{-1}, respectively. In contrast, plants LGM11 and LGM06 exhibited lower flavonoid levels, with LGM11 showing the lowest concentration, approximately 20 mg QE · gfw^{-1}, with no significant differences compared to the control. These findings suggest that mutagenesis in certain genotypes enhanced flavonoid accumulation, which was potentially linked to an increase in secondary metabolite production as a response to induced genetic changes.

The total phenolic content (TPC), expressed in milligrams of gallic acid equivalents per gram of fresh weight (mg GAE · gfw^{-1}), was evaluated in *Lippia graveolens*, including a non-mutagenized control (CTL) and six mutant lines (LGM06, LGM11, LGM14, LGM20, LGM23, and LGM25) (Fig. 4).

Statistical analysis revealed significant differences ($p < 0.05$) in TPC between the treatments, as indicated by distinct letters above the bars. The mutant line LGM25 exhibited the highest TPC, significantly exceeding all other plants. In contrast, LGM11 displayed

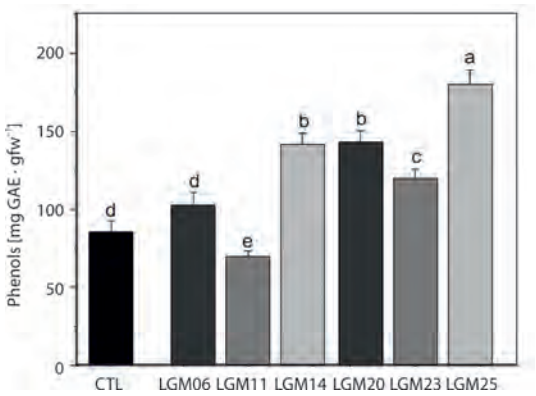

Fig. 4. Quantitative determination of total phenolics content (TPC) of *L. graveolens* mutants. Means of the TPC for three replicates and standard deviations are shown. Different letters above the bars indicate significant differences ($p < 0.05$). Control (CTL) corresponds to non-mutagenized oregano plants

the lowest TPC. These results indicate that mutagenesis can induce substantial variations in the phenolic content of *L. graveolens*, highlighting its potential as a tool for enhancing phenolic compound levels in this species.

Discussion

Ethyl methanesulfonate (EMS) is a potent alkylating agent that induces high-frequency random chemical modifications in nucleotides, particularly guanine, which often results in point mutations by converting GC base pairs into AT pairs. This mutagen has been widely used to generate novel plant varieties, exhibiting both morphological and physiological changes. It has proven effective in improving agronomic traits such as drought tolerance (Khalil *et al.* 2018; Chen *et al.* 2023). While seeds are the most commonly used plant material for chemical mutagenesis, EMS has also been successfully applied to various explants used in *in vitro* vegetative propagation (Mullins *et al.* 2021). Previous work has demonstrated the effectiveness of EMS in promoting genetic diversity in *Lippia graveolens* through mutagenesis of nodal explants from a single *in vitro*-propagated genotype. This approach made it possible to assess genetic variation using molecular markers, confirming the potential of this technique for the genetic improvement of *L. graveolens* (Muñoz-Miranda *et al.* 2019).

In this study, the aim was to generate drought-tolerant mutants using seeds as the starting material for mutagenesis. It is commonly considered that a mutagen dose causing a lethality rate of 50% is appropriate for mutagenesis experiments, as it may result in the highest mutation rates (Chen *et. al.* 2023). Based on the observed reduction in germination rate and the number of putative mutants obtained, 0.1% EMS for 1 hr would be a suitable treatment for mutagenesis of *L.* graveolens seeds. Both polyethylene glycol (PEG) and mannitol were employed to simulate drought conditions. Initial tests revealed that direct germination of *L. graveolens* seeds on media supplemented with these compounds significantly inhibited germination. Therefore, post-mutagenesis seeds were germinated in osmolyte-free MS medium, and potential mutants were selected during micropropagation using nodal explants derived from the seedlings.

During the optimization of osmolyte concentrations, it was observed that mannitol did not significantly impact explant development or shoot regeneration. This rendered it unsuitable as a selective agent. Mannitol's protective role against water stress, as an antioxidant, has been proposed in some plants. It is thought to mitigate reactive oxygen species (ROS) formation under drought stress by scavenging hydroxyl radicals, providing protection against abiotic stresses such as salt and drought (Patel and Williamson 2016). This phenomenon could explain the minimal impact of high mannitol concentrations observed in *L. graveolens*. Conversely, PEG was highly effective in inducing drought-like conditions, particularly at 50 $g \cdot l^{-1}$, which caused the death of wild-type plants. Therefore, PEG was used to screen mutants under selective pressure. The reliability of PEG for mimicking drought has been debated because, despite causing some typical plant responses to drought stress, impairments in nutrient uptake and alterations in carbon metabolism have been observed compared to plants with reduced irrigation (Castañeda and Gonzalez 2021; Gonzalez *et al.* 2024). However, this compound remains useful for *in vitro* screening of EMS-induced drought-tolerant mutants, which maintain this tolerance when subjected to water deficit conditions in greenhouse pot trials (Khalil *et al.* 2018; Tiryaki *et al.* 2022).

Although EMS exposure significantly reduced germination rates, in some cases to less than 10%, the surviving seedlings were sufficient for the selection of potential mutants. The reduced germination observed with EMS can be attributed to chromosomal deletions, mitotic suppression, and other dose-dependent biochemical and physiological effects (Singh *et al.* 2021). Selected plants that survived the micropropagation process displayed noticeable morphological changes, suggestive of genetic alterations. The total flavonoid and phenol content in these mutants was assessed, as these compounds play crucial roles in defense against abiotic and biotic stresses. Under drought conditions, plants produce ROS, leading to protein degradation, lipid peroxidation, and cellular damage. The phenylpropanoid pathway, which synthesizes phenolic and flavonoid compounds, helps mitigate ROS accumulation,

thus protecting the plant (Chowdhary *et al.* 2022; Tiryaki *et al.* 2022).

Interestingly, there was no direct correlation between drought tolerance in the mutants and increased flavonoid or phenolic content, as some mutants exhibited lower levels of these compounds than non-mutagenized plants. However, the positive correlation observed between flavonoid and phenolic content in the mutants suggests that certain mutations may have affected shared regulatory points in the phenylpropanoid pathway. It is possible that mutants with lower levels of phenolic compounds and flavonoids possess other mechanisms to cope with the oxidative stress caused by water deficiency, such as the accumulation of other metabolites with antioxidant activity, including terpenes, glutathione, ascorbate, or the increased activity of enzymatic antioxidants like superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT), among others (Mishra *et al.* 2023; Haghpanah *et al.* 2024).

Further analysis of gene expression in mutant plants, particularly genes encoding the enzymes or transcription factors involved in the biosynthesis and regulation of these compounds, would provide valuable insights into the mechanisms of drought tolerance in this species. Unfortunately, the *L. graveolens* genome has not been fully sequenced, and current information on gene sequences is limited. Ongoing work to sequence the transcriptome of *L. graveolens* will provide key data on genes involved in drought responses, making it possible to evaluate their expression in mutant plants. Additionally, it will be crucial to assess the field performance of these mutants by subjecting them to drought conditions under more realistic environmental scenarios.

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ORIGINAL ARTICLE

Assessment of some herbicide effects on the weed control and agronomic traits of *Camelina sativa* under irrigation and rainfed conditions

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Abstract

Climatic fluctuations in semi-arid areas are modifying the dynamics of weeds in field crops production. This trial was aimed to research the application of various herbicides on the growth characteristics of camelina under irrigation and rainfed conditions in Maragheh, Iran. Analyzing the data via treatment by a trait biplot model based on principal component (PCA) indicated that the PCA1 and PCA2 explained 74 and 17% of the observed variability, "respectively". Most traits were grouped under the I1-H3 (irrigated + cycloxydim) section while the number of siliques per plant was positioned with I1-H5 (irrigated + pinoxaden), and I1-H1 (irrigated + without herbicide) produced a high harvest index as well as density and biomass of weeds. The seed yield, biological yield, thousand-seed weight, seed number per silique, canopy diameter, chlorophyll content, plant height, and the number of siliques per plant were correlated similar to association of weed density with biomass. Herbicides H3, H4 (haloxyfop-R-methyl), and H5 were positively correlated under irrigated conditions, while H2 (trifluralin), H3, H4, and H5 were positively associated under rainfed circumstances. The number of siliques per plant, seeds per silique, and seed yield demonstrated high discriminative ability as well as greater typical ability. Treatments H2, H4 and H5 under irrigation conditions were identified as ideal or distinguishing treatments. The response of treatments for seed yield indicated that H3, H4 and H5 under full-irrigated conditions were the most effective in terms of yield performance. Overall, this study underscored that the availability of water strongly affected the effectiveness of herbicides, thus, it seems necessary to use climate smart agricultural practices in drought prone regions such as supplemental irrigation to improve the effectiveness of acetyl CoA carboxylase inhibitor herbicides.

Keywords: cycloxydim, haloxyfop-R-methyl, herbicide efficacy, pinoxaden, treatment by trait interaction

Introduction

Water scarcity is a key challenge for crop productivity in semi-arid regions, worsened by climate change and rising temperatures. *Camelina sativa*, a resilient oilseed crop from the Brassicaceae family, has regained interest due to its ability to produce reasonable yields with minimal inputs (Sydor *et al.* 2022). It is highly resistant to pests, diseases, drought, heat, and salinity. However, weed competition remains a major issue, impacting net productivity. Traditional crop breeding has prioritized yield over stress tolerance, making effective weed management crucial (Cierjacks *et al.* 2016). While camelina cultivation is increasing in semi-arid regions, research on herbicide efficacy in its production remains limited, highlighting the need for studies on optimizing weed control strategies to enhance yield potential.

Semi-arid climates present significant challenges to plant growth, including limited and irregular rainfall, low winter temperatures at higher altitudes, nutrient deficiencies, high soil pH, and prolonged dry spells

(Fattahi *et al.* 2023; Janmohammadi *et al.* 2024). Most precipitation occurs during the cold months when plants are in the inactive rosette stage, further restricting growth. While some studies have examined the effects of water scarcity on camelina, showing declines in vegetative growth, seed yield, and oil quality, little research has explored how drought affects herbicide efficacy in camelina cultivation. Pre-plant herbicides, like quinclorac and pendimethalin, have been tested, revealing that camelina's early growth stage is highly sensitive to these soil-applied herbicides. However, as the plant matures, the negative effects diminish, with quinclorac proving to be the safest option (Jha and Stougaard 2013). Further research is needed to optimize herbicide use under water-limited conditions to support camelina production in semi-arid regions.

Propaquizafop and quizalofop-p-ethyl are systemic herbicides absorbed through both foliage and roots, transported via the phloem, and act by inhibiting fatty acid synthesis through acetyl-CoA carboxylase (ACCase) (Jursík *et al.* 2021). Testing these herbicides on different camelina varieties revealed varying effects on chlorophyll fluorescence and photosystem II function (Sobiech *et al.* 2020). In contrast, clopyralid and picloram, selective herbicides targeting broadleaf weeds and woody plants by mimicking auxin, had minimal influence on camelina's photosynthetic performance (Sobiech *et al.* 2020). Under water-deficit conditions, herbicides such as clodinafop-propargyl and mesosulfuron-methyl + iodosulfuron-methyl sodium showed significantly reduced effectiveness (Alizade *et al.* 2021), likely due to decreased stomatal conductance limiting herbicide absorption. While some studies have examined specific herbicides in camelina cultivation, their performance under different moisture conditions remains largely unexplored, highlighting the need for further research. Due to the dominance of cereal cultivation in the semi-arid regions of northwestern Iran, many narrow-leaved weeds are prevalent in these areas. With the introduction of camelina to these areas, there is a need to evaluate common herbicides, especially ACCase inhibitors types in production systems. This study aimed to assess the effects of trifluralin and the post-emergence herbicides cycloxydim, haloxyfop-R-methyl, and pinoxaden on weed control and camelina seed yield components under well-irrigated and rainfed conditions.

Materials and Methods

Trial

A field trial was conducted in Maragheh, Iran (37°23'N,46°16'E) during the 2023–2024 cropping season. This region receives most of its precipitation in winter, with a total of 294 mm recorded from September to July, approximately 70% of which falls as snowfall. The field's soil is silty loam with a pH of 7.8 and key properties including low organic matter (0.3%), nitrogen (0.1%), calcium carbonate (11%), electrical conductivity (0.92 $dS \cdot m^{-1}$), phosphorus (11.3 $mg \cdot kg^{-1}$), and potassium (282 $mg \cdot kg^{-1}$). The field remained fallow the previous season. In autumn, primary tillage was performed using a moldboard plow, incorporating 10 tons per hectare of farmyard manure. In early March, secondary tillage followed with a disc harrow, rotavator, and leveler. The soil was then shaped into ridges and furrows using a Hiller-Furrower, with ridges spaced 50 cm apart, 10 cm wide at the top, and 15 cm high. Before planting, phosphorus (60 kg as triple superphosphate) and nitrogen (100 kg as urea) were applied per recommended rates. In mid-March, the field was divided into main and sub-plots for experimentation. Camelina seeds (Sohail cultivar) were obtained from the Dryland Agricultural Research Institute, Iran. This variety is highly adaptable to diverse climates and soils, demonstrating superior drought tolerance compared to other oilseed crops (Kahrizi *et al.* 2018). It requires less water, fertilizer, and pesticides while exhibiting strong cold resistance. The seeds were manually planted on ridge tops at a depth of 1.5 cm, with 5 cm spacing between rows. Sowing coincided with the start of the rainy season, allowing natural rainfall to support germination and seedling establishment.

Treatments

The experiment followed a split-plot design (2 × 5) within a randomized complete block framework, with three replications. The main factor included two irrigation regimes: I1 (irrigated) and I2 (rainfed). In the irrigated treatment, the field received eight irrigation events from planting to harvest, totaling 550 mm of water. Sub-plots were assigned to five herbicide treatments including H1, no herbicide application (control); H2, trifluralin (pre-emergence); H3, cycloxydim (post-emergence); H4, haloxyfop-R-methyl (post-emergence); and H5, pinoxaden (post-emergence). Trifluralin (Treflan, CAS 1582-09-8) was applied at $1.5\,l \cdot ha^{-1}$ 10 days before planting, following plowing and disc operations, and was incorporated into the soil using a light disc. This herbicide inhibits microtubule synthesis, disrupting mitosis and preventing weed seedling germination and growth. Cycloxydim is a systemic, selective herbicide from the cyclohexane oxime group, specifically targeting narrow-leaved weeds in canola crops. It is applied at $2\,l \cdot ha^{-1}$ post-emergence, once narrow-leaved weeds reach the four- to five-leaf stage.

Haloxyfop-R-methyl (Galant Super, EC 10%, produced by Mehr Parsian Exir Co., Iran) is a systemic, selective herbicide effective against a wide range of

annual and perennial narrow-leaved weeds. It is applied at a rate of 1 l per hectare when the weeds are at the two- to five-leaf stage. Belonging to the aryloxyphenoxypropionate group, haloxyfop-R-methyl is absorbed by plants within 1 hour of application. Pinoxaden (Axial, EC 5%, produced by Aria Shimi, Iran) is the only herbicide available in the pinoxaden group, targeting and inhibiting the growth of narrow-leafed grasses. It was applied when the weeds had four to six leaves, at a rate of 1.2 l per hectare. In the control treatment, no herbicide was applied, and the weeds were sprayed with distilled water. All herbicides were applied following the recommended protocols during the weeds' vegetative stage using a motorized backpack sprayer (Solo model 433). Post-emergence herbicides were applied to the above-ground parts of the weeds early in the day, ensuring complete coverage of the weed shoots without runoff.

Traits

Following the application of post-emergence herbicides and at the midpoint of the main stem elongation phase, surviving weeds between and within the rows were counted using a 1 m^2 quadrat in each experimental plot. Weed population density was recorded as the number of weeds per square meter. The weeds were then cut above the ground, treated at 70°C for 48 hours in an oven to determine weed dry biomass. At the early flowering stage, chlorophyll content in the upper, fully developed leaves of camelina was measured using a SPAD 502 chlorophyll meter (Konica Minolta, USA). At physiological maturity, plant height was measured from a 1 m^2 quadrat randomly taken from each plot, and canopy diameter was assessed by measuring from left to right to evaluate lateral canopy growth. After harvesting and drying the camelina plants, seed yield components were calculated, including the number of siliques per plant, seeds per silique, 1000-seed weight, yield per unit area, biological yield, and the harvest index.

Biplot analysis

The entry-by-tester biplot model, implemented through the GGEbiplot application (Yan 2024), is a powerful tool for analyzing datasets, including those derived from various herbicide and irrigation treatments. This model is based on principal component analysis (PCA) and facilitates the visualization and interpretation of interactions between entries (e.g., treatments) and testers (e.g., traits) using the following equation:

$$\frac{Y_{ij}-\bar{Y}_J}{\sqrt{S_j^2}} = \sum_{n=1}^{2} \Phi_n \Psi_{in} \Omega_{jn} + R_{ij},$$

where:

Y_{ij} is the average of treatment i for trait;

$\bar{Y}_J$ is the means Y_{ij}; S^2_j is the variance;

Φ_n is the eigen-value for PCA *n*;

Ψ_{in} is score of entry i on PCA *n*;

Ω_{jn} is score of tester j on PCA *n*;

R_{ij} is the unaccounted variability of the computed equation.

To obtain symmetrical scores for both treatments and traits, ensuring their comparability, the Φ value is transformed through vector absorption, leading to a more precise representation of treatments and traits. The entry-by-tester interaction biplots are generated using score scales, where each entry or tester is represented as a distinct point on the plot. By applying this model, valuable insights are gained into the effects of herbicides and irrigation on camelina, providing a foundation for optimizing future agricultural practices.

Results and Discussion

Model fitting

The first and second principal components (PCs) collectively explained 91% of the total variability (Fig. 1), with the first PC accounting for 74% and the second PC for 17%. This variation in the entry-by-tester interaction underscores the substantial influence of both additive and crossover effects, which contribute to shifts in treatment rankings across traits. These findings align with those of Sabaghnia and Janmohammadi (2016) in sunflower, who emphasized the challenges of selecting optimal treatments without considering entry-by-tester interactions. Therefore, the application of the biplot method is strongly recommended to effectively address these complexities. Similarly, Yari *et al.* (2018) demonstrated that the entry-by-tester biplot model serves as a robust analytical tool for examining the relationships between treatments and traits.

Polygon-view

Figure 1 provides a visual representation of the impact of herbicide and irrigation treatments, identifying treatment combinations that were most effective for specific traits. Several agronomic traits, including seed yield (SY), biological yield (BY), thousand-seed weight (TSW), seed number per silique (SNS), canopy diameter (CD), chlorophyll content (CC), and plant height (PH), were clustered under the I1-H3 treatment (irrigation + cycloxydim). Our results indicated that cycloxydim, when applied under optimal soil moisture conditions, exerted a more pronounced positive effect on both vegetative and reproductive growth in

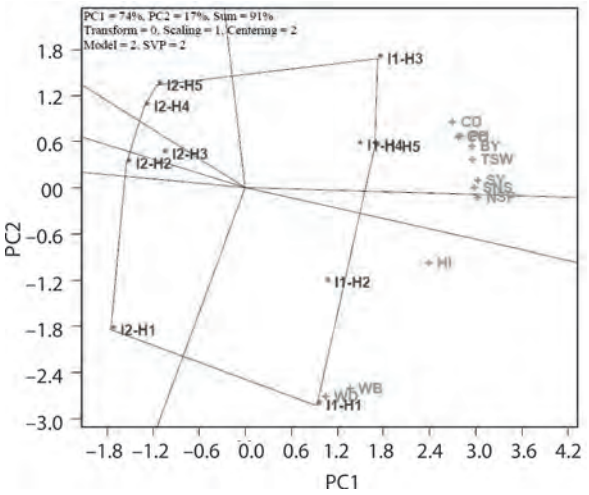

Fig. 1. A heptagon of a biplot model showing the reaction of camelina traits under various treatments. Treatments are: I1 – irrigated conditions; I2 – rainfed circumstances; H1 – no application of herbicide; H2 – trifluralin; H3 – cycloxydim; H4 – haloxyfop-R-methyl; and H5 – pinoxaden. Traits are: WD – weed density; WB – weed biomass; HI – harvest index; SY – seed yield; BY – biological yield; TSW – thousand-seed weight; SNS – seed number per silique; NSP – number of siliques per plant; CD – canopy diameter; CC – chlorophyll content; and PH – plant height

camelina. As a selective herbicide targeting grasses, cycloxydim is less detrimental to crops than the broad-spectrum herbicide trifluralin, even when applied during active growth. Moreover, the findings suggest that irrigation plays a critical role in herbicide efficacy. Under drought conditions, reduced herbicide mobility within vascular systems, lower absorption rates, and altered herbicide metabolism in weeds, resulting from oxidative stress, can significantly impair herbicidal effectiveness (Alizade *et al.* 2020).

The number of siliques per plant (NSP) was positioned separately, with the I1-H5 treatment (irrigated + pinoxaden) being the most effective for this trait. The superior performance of pinoxaden compared to other herbicides under irrigation conditions in improving camelina growth can be attributed to its ability to eliminate weeds during reproductive stages, thereby reducing competition between weeds and camelina. The lack of effectiveness of pinoxaden under rainfed conditions is likely due to physiological and anatomical adaptations in weeds, including xenobiotic compartmentalization and detoxification mechanisms such as hydroxylation or dealkylation mediated by cytochrome P450 (Kulasekaran *et al.* 2017; Yanniccari *et al.* 2020). Furthermore, the I1-H1 treatment (irrigated + no herbicide) was associated with high values for weed density (WD), weed biomass (WB), and harvest index (HI). In contrast, the four remaining vertex treatments, I2-H1, I2-H2, I2-H3, and I2-H4 (rainfed + no herbicide, trifluralin, haloxyfop-R-methyl, and pinoxaden, respectively), performed poorly across all measured traits. These findings suggest that camelina yield is primarily influenced by soil moisture availability, and that weed control under rainfed conditions is less effective than under irrigation. Among the herbicide treatments, cycloxydim (H3) produced the highest yield and yield components, demonstrating greater efficacy than the other applied herbicides. The fitted entry-by-tester biplot model effectively highlighted variations in camelina traits under different irrigation and herbicide treatments, facilitating the identification of the most optimal treatment combination. Therefore, for camelina cultivation in semi-arid regions, farmers should consider appropriate irrigation practices and weed control strategies, particularly using cycloxydim, to achieve optimal results. One potential factor that could reduce the effectiveness of cycloxydim is photolysis on leaf surfaces. Previous studies indicate that increased accumulation of waxes on leaf cuticles accelerates the photolysis rate of this herbicide (Xi *et al.* 2022). However, under favorable humidity conditions, reduced wax deposition on the cuticle appears to enhance herbicide penetration and efficacy in weed control (Monadjemi *et al.* 2014).

This experiment revealed that irrigation conditions played a significant role in determining the effectiveness of herbicides in camelina cultivation, with irrigated treatments generally showing better results in terms of weed control and crop yield. Irrigated conditions (I1) supported better herbicide absorption and activity because the higher soil moisture facilitated the movement of herbicides within the soil and plants. This meant that when camelina was irrigated, herbicides like cycloxydim (H3) worked more effectively, controlling weeds and allowing the crop to thrive. Under these conditions, cycloxydim resulted in enhanced vegetative and reproductive growth, contributing to better traits like seed yield, biological yield, thousand-seed weight, canopy diameter, and chlorophyll content. Cycloxydim is a selective herbicide for grasses, and its systemic action works better under moist conditions, as it can be absorbed more efficiently by both the foliage and the roots. In contrast, under rainfed conditions (I2), where water availability was limited, herbicides showed reduced effectiveness. This was particularly true for trifluralin (H2), haloxyfop-R-methyl (H4), and pinoxaden (H5), where the lack of moisture likely hindered their movement in the plants' vascular systems. Decreased absorption of these herbicides under dry conditions meant that they could not work as efficiently to control weeds. Furthermore, drought-induced oxidative stress could alter how weeds metabolize herbicides, possibly leading to detoxification and reduced herbicide efficacy.

Cycloxydim (H3), applied post-emergence, was the most effective herbicide under irrigated conditions. It was particularly beneficial for controlling grasses

and promoting camelina growth by reducing weed competition. Cycloxydim does not damage camelina crops, even if applied slightly over the recommended dose during active growth, making it a safer choice for camelina cultivation. Pinoxaden (H5), also a post-emergence herbicide, was most effective for controlling weeds under irrigated conditions. This herbicide was particularly beneficial for the number of number of siliques per plant (NSP), which suggests its positive impact during the reproductive stages of camelina. Pinoxaden works by inhibiting ACCase in grass weeds, but its effectiveness was diminished under rainfed conditions. The lower efficacy of pinoxaden under rainfed conditions could be linked to physiological changes in the weeds, such as detoxification mechanisms (e.g., cytochrome P450-mediated detoxification), which might reduce herbicide uptake and effectiveness in stressed, drought-affected weeds. Trifluralin (H2) and haloxyfop-R-methyl (H4), both pre- and post-emergence herbicides, showed reduced efficacy under rainfed conditions compared to irrigated ones. Trifluralin, which inhibits microtubule formation in weeds, was particularly ineffective when water was scarce, as it relies on soil incorporation and effective root absorption. Similarly, haloxyfop-R-methyl, an herbicide targeting broadleaf weeds and some grasses, worked better under irrigated conditions, where its absorption and translocation through plants were more efficient. Under rainfed conditions, these herbicides had limited success due to lower herbicide movement and reduced absorption in dry soils.

The efficacy of herbicides under irrigated conditions was largely determined by their systemic nature and how well they were absorbed and transported within the plant system. Cycloxydim and pinoxaden both showed high efficacy in reducing weed density and promoting camelina growth, as they could move efficiently through the plant under favorable moisture conditions. In contrast, broad-spectrum herbicides like trifluralin did not perform as well under both irrigated and rainfed conditions, as they tend to affect a wide range of weeds and have a more severe impact on soil health and crop safety, potentially hindering camelina growth. Weed competition was notably reduced under irrigated conditions, which might explain the improved number of siliques per plant and other yield components in treatments where cycloxydim and pinoxaden were used. These herbicides helped control weeds during the crucial reproductive phase, reducing competition and allowing camelina to allocate more resources to seed development. However, under rainfed conditions, where weeds are harder to control due to limited herbicide efficacy, weed competition became a major limiting factor for camelina growth and seed yield.

Traits' associations

The correlation among testers, as illustrated in Figure 2A, depicts the relationships between the measured traits of camelina. The cosine of the angles formed by the trait vectors relative to the origin indicates the strength and direction of these relationships. Traits are positively correlated when their vectors form an angle smaller than 90°, negatively correlated when the angle exceeds 90°, and unrelated when the angle is exactly 90°. In Fig. 2A, weed density (WD) and weed biomass (WB) exhibit a strong positive correlation, as indicated by their small acute angles. Similarly, other agronomic traits, including seed yield (SY), biological yield (BY), thousand-seed weight (TSW), seed number per silique (SNS), canopy diameter (CD), chlorophyll content (CC), plant height (PH), and the number

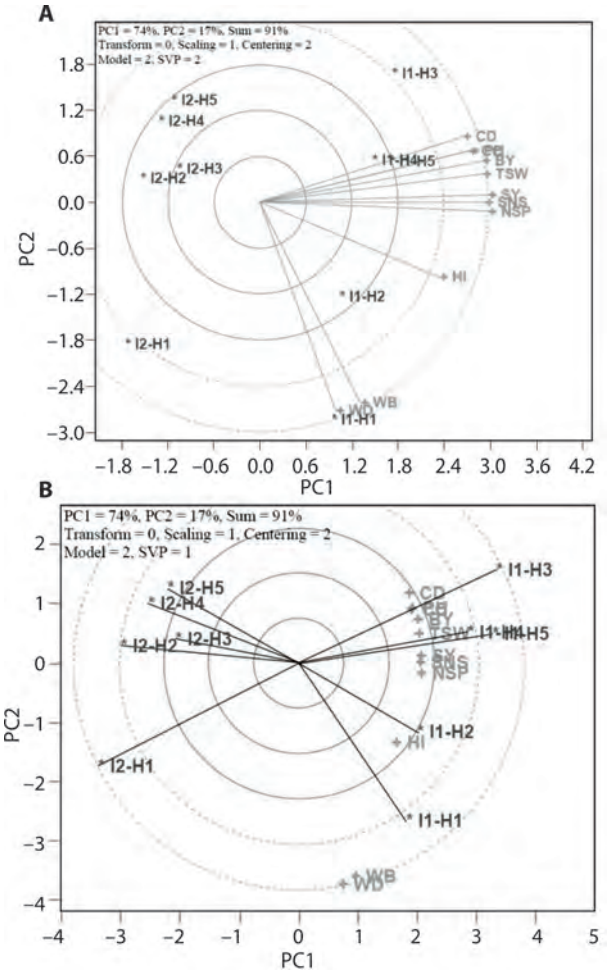

Fig. 2. Vector-option of biplot model showing relations between A – traits and B – treatments in camelina. Treatments are: I1 – irrigated conditions; I2 – rainfed circumstances; H1 – no application of herbicide; H2 – trifluralin; H3 – cycloxydim; H4 – haloxyfop-R-methyl; and H5 – pinoxaden. Traits are: WD – weed density; WB – weed biomass; HI – harvest index; SY – seed yield; BY – biological yield; TSW – thousand-seed weight; SNS – seed number per silique; NSP – number of siliques per plant; CD – canopy diameter; CC – chlorophyll content; and PH – plant height

of siliques per plant (NSP), also show positive correlations, as reflected by their acute angles. In contrast, the correlations between weed density (WD) and weed biomass (WB) with all other agronomic traits, except for harvest index (HI), were close to zero, as indicated by the approximately 90° angles. The entry-by-tester interaction biplot model's predictions regarding trait relationships closely align with the numerical correlations presented in Table 1. However, minor discrepancies may exist due to the model explaining 91% of the variation rather than a complete 100%. Previous research by Fallah *et al.* (2023) also demonstrated that camelina yield performance is strongly associated with biomass, thousand-seed weight, seed number per silique, plant height, and the number of siliques per plant. The analysis of trait correlations in Figure 2A reinforced the fact that camelina's productivity is influenced by multiple interrelated factors, with weed control playing a crucial role, especially under irrigated conditions. Managing these correlated traits, particularly by controlling weeds through effective herbicide use, can significantly improve camelina's growth and yield.

Treatments' associations

Similarly, the correlation among entries, as illustrated in Figure 2B, highlights the associations between treatments. Under irrigated conditions, I1-H3, I1-H4, and I1-H5 exhibited a positive correlation, as indicated by their small acute angles. These findings confirm that under full irrigation, aryloxyphenoxypropionate herbicides demonstrated comparable efficacy in reducing competitive pressure from narrow-leaved weeds. The probable mechanism behind this effect was the suppression of weed competition, which enhances camelina growth by improving photoassimilate production, increasing soil nutrient availability, optimizing soil moisture conditions in the rooting zone, and enhancing light absorption by the crop canopy. Ghidoli *et al.* (2023) reported that with optimal agricultural management and improved establishment of *C. sativa* under favorable moisture conditions, the plant can effectively compete with weeds by expanding its canopy and covering the soil surface.

The treatments under rainfed conditions (I2-H2, I2-H3, I2-H4, and I2-H5) also exhibited positive correlations, as indicated by their acute angles. Conversely, I1-H3 demonstrated a negative relationship with I2-H1, while I1-H2 showed a negative association with I2-H4 and I2-H5, as reflected by their obtuse angles (Fig. 2B). Similarly, I2-H2 was negatively correlated with I1-H4 and I1-H5, as indicated by their obtuse angles. These results highlight a significant distinction between irrigated and rainfed treatments, although the performance of H4 (haloxyfop-R-methyl) and H5 (pinoxaden) remained consistent across both conditions. Under irrigation, notable differences were observed between H2 (trifluralin) and H3 (cycloxydim) compared to H4 and H5. However, under rainfed conditions, these differences diminished, and all treatments, except for the control, exhibited relatively similar responses. It is important to note that the continuous use of ACCase-inhibiting herbicides has led to an increasing occurrence of resistance in certain narrow-leaved weed species, including *Avena sterilis*, *Avena fatua*, *Phalaris minor*, *Phalaris paradoxa*, and *Lolium rigidum*, particularly in northwestern Iran (Gherekhloo *et al.* 2016). Under rainfed conditions, herbicides H4 (haloxyfop-R-methyl) and H5 (pinoxaden) were effective across all treatments, showing consistent performance in weed control and agronomic traits. Under irrigated conditions, cycloxydim and trifluralin performed better than the rainfed, with cycloxydim showing a positive correlation with several growth traits. Herbicide efficacy is strongly influenced

Table 1. Associations among traits of camelina under various herbicides and water regimes

	WD	WB	PH	CH	CD	NSI	SNS	TSW	BYC	SYC
WB	0.920									
PH	0.138	0.212								
CH	0.063	0.205	0.803							
CD	0.068	0.136	0.852	0.795						
NSI	0.365	0.482	0.932	0.871	0.848					
SNS	0.314	0.421	0.905	0.874	0.845	0.977				
TSW	0.205	0.325	0.950	0.916	0.840	0.967	0.928			
BYC	0.190	0.281	0.942	0.892	0.941	0.954	0.934	0.946		
SYC	0.290	0.398	0.886	0.919	0.900	0.973	0.967	0.954	0.956	
HIC	0.469	0.564	0.530	0.694	0.564	0.747	0.765	0.702	0.601	0.808

Significant levels are 0.63 and 0.77 at 0.05 and 0.01 statistical level in DF = 8. Traits are: WD – weed density; WB – weed biomass; HI – harvest index; SY – seed yield; BY – biological yield; TSW – thousand seed weight; SNS – seed number per silique; NSP – number of siliques per plant; CD – canopy diameter; CC – chlorophyll content; and PH – plant height

by irrigation, with cycloxydim and trifluralin showing reduced effectiveness under rainfed conditions. Haloxyfop-R-methyl and pinoxaden showed similar positive effects under both conditions, suggesting that they are reliable choices for both rainfed and irrigated camelina cultivation. The issue of herbicide resistance in certain weed species emphasizes the need for effective management strategies to ensure sustainable weed control and maintain the efficacy of herbicides.

Ideal trait

The discriminative ability of a trait is reflected in its standard deviation, with a higher ability indicated by a closer proximity to the ideal trait position (Fig. 3A). Traits closer to this ideal position are considered more favorable, while those positioned farther from it are less effective. In this study, traits such as the number of siliques per plant (NSP), seed number per silique (SNS), and seed yield (SY) demonstrated high discriminative ability. These were followed by harvest index (HI), biological yield (BY), thousand-seed weight (TSW), chlorophyll content (CC), and plant height (PH). All traits exhibited discriminative ability above the mean level, enabling them to effectively differentiate between the treatments (Fig. 3A). The typical ability of a trait, which reflects how well it represents the characteristic, is determined by the angle between the trait's vector and the horizontal axis (which represents the average of all traits). A smaller angle indicates greater typical ability. Consequently, the more favorable traits; NSP, SNS, and SY, had smaller angles with the horizontal axis, indicating higher typical ability. In contrast, canopy diameter had a larger angle, reflecting relatively lower typical ability (Fig. 3A).

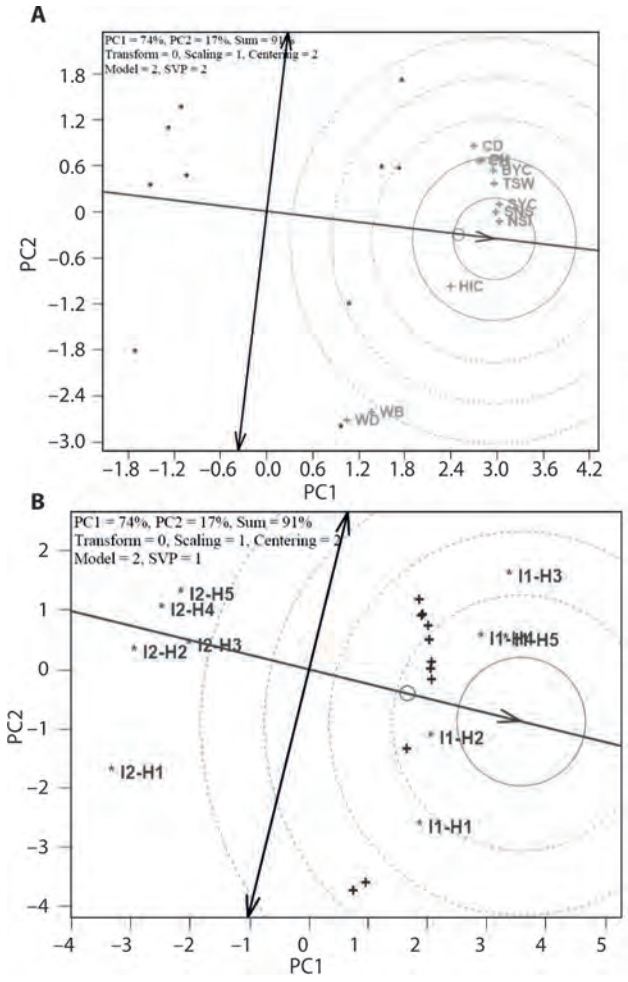

Fig. 3. A – perfect trait and B – perfect treatment tool of biplot model showing the position of the perfect trait and treatment in camelina. Treatments are: I1 – irrigated conditions; I2 – rainfed circumstances; H1 – no application of herbicide; H2 – trifluralin; H3 – cycloxydim; H4 – haloxyfop-R-methyl; and H5 – pinoxaden. Traits are: WD – weed density; WB – weed biomass; HI – harvest index; SY – seed yield; BY – biological yield; TSW – thousand-seed weight; SNS – seed number per silique; NSP – number of siliques per plant; CD – canopy diameter; CC – chlorophyll content; and PH – plant height

Ideal treatment

The potential for distinguishing treatments based on traits and their typical ability to highlight key characteristics is represented by an idealized treatment, known as the perfect position (Fig. 3B). The most effective treatments are those closest to this ideal position, while those farther from it are considered less desirable. For example, I1-H2, I1-H4, and I1-H5, followed by I1-H1 and I1-H3, are considered ideal treatments as they were closest to the perfect position. In contrast, I2-H1, followed by I2-H2, I2-H3, I2-H4, and I2-H5, were positioned farther from the ideal, making them the least desirable treatments in terms of both their distinguishing and typical abilities. Interestingly, all the ideal treatments were from irrigated conditions, indicating that the distinguishing potential of treatments was more evident under normal conditions. However, under stressful conditions, this ability decreased significantly. These ideal treatments can be viewed as ideotypes, demonstrating high performance across most traits. Nonetheless, challenges arise when associations between traits are not always significant. This issue is particularly relevant in camelina production, where both yield performance and quality characteristics are critical for achieving optimal outcomes. In this context, the use of multivariate statistical models with visual outputs becomes increasingly valuable for identifying the best treatments. Ultimately, the most effective treatments identified were I1-H2, I1-H4, and I1-H5, which involved the application of trifluralin, haloxyfop-R-methyl, and pinoxaden herbicides under irrigated conditions. In summary, irrigated treatments (I1-H2, I1-H4, and I1-H5) were the most effective, exhibiting strong correlations with high performance in traits like seed yield, thousand-seed weight, and plant

height. Rainfed treatments showed reduced discriminative ability, with herbicides being less effective under these conditions due to lower herbicide absorption and reduced effectiveness of narrow-leaved weed control.

Yield evaluation

The effects of treatments on seed yield (SY) are illustrated in Figure 4A, where an axis representing SY is indicated with an arrow in its direction. Treatments I1-H3, I1-H4, and I1-H5, followed by I1-H2 and I1-H1, were the most effective for achieving high yield performance. In contrast, treatments I2-H1, followed by I2-H2, I2-H3, I2-H4, and I2-H5, were the least favorable for seed yield performance (Fig. 4A). It appears that the amount of available soil moisture influenced the effectiveness of herbicides by altering the morphological characteristics of crop plants. These changes can affect herbicide absorption, translocation efficiency, metabolism of toxic herbicide compounds (through the addition of functional groups), and the nature of the weed population, including weed biodiversity, plant density, and the dominance of specific weed species (Peerzada *et al.* 2021). The distance of treatments from the SY axis reflects the standard deviation, with smaller intervals indicating better consistency. Consequently, I1-H4 and I1-H5 exhibited smaller intervals and showed less variation, making them more reliable choices. In contrast, I2-H1 had lower seed yield performance (below the average, represented by the blue axis) and a larger interval from the SY axis, indicating greater variation, making it one of the least favorable treatments. The application of haloxyfop-R-methyl (H4) and pinoxaden (H5) under irrigated conditions led to an increase in seed yield, emphasizing the importance of effective weed control through the use of appropriate herbicides, as well as providing a suitable water supply. Considering the agro-climatic zone of the studied area and the common use of cycloxydim in onion fields, these two herbicides, haloxyfop-R-methyl and pinoxaden, are suitable options for herbicide rotation to prevent the emergence and spread of resistance to ACCase-inhibiting herbicides.

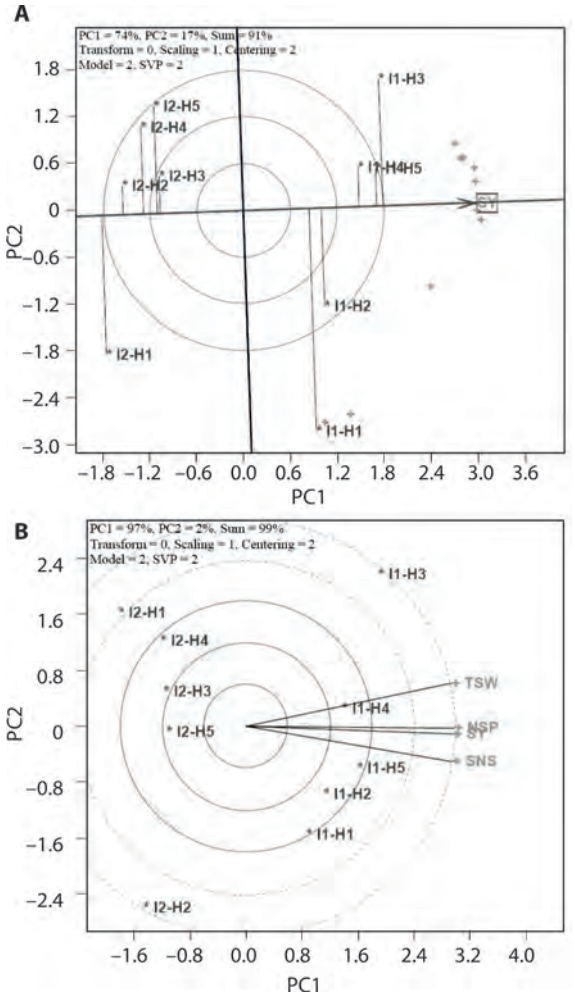

Fig. 4. A – reaction of various of treatments for seed yield (SY) performance and B – vector-option of biplot model showing relations between yield components in camelina. Treatments are: I1 – irrigated conditions; I2 – rainfed circumstances; H1 – no application of herbicide; H2 – trifluralin; H3 – cycloxydim; H4 – haloxyfop-R-methyl; and H5 – pinoxaden. Traits are: SY – seed yield; TSW – thousand-seed weight; SNS – seed number per silique; NSP – number of siliques per plant

Yield components

For camelina, three yield components are particularly important: thousand-seed weight (TSW), seed number per silique (SNS), and the number of siliques per plant (NSP). The related vectors display the relationships between these yield components (Fig. 4B), where seed yield is positively correlated with these components, especially the number of siliques per plant. Berti *et al.* (2011) identified the number of siliques per plant and thousand-seed weight as the most influential yield components in camelina, which is considered a promising alternative crop for farmers in South Central Chile. Similarly, Jiang and Caldwell (2016) found that seed yield in camelina was associated with the number of siliques per plant, thousand-seed weight, and siliques per unit area. Although the herbicides used in this study were effective in improving the growth and performance of camelina by controlling narrow-leaf weeds, the growth of broad-leaved weeds, such as herb--Sophia, shepherd's-purse, wild mustard, bastard cabbage, prickly lettuce, field bindweed, common fumitory, catchweed, and lady's thumb, remains a limiting factor for camelina growth. Therefore, the combined use of broadleaf and narrow-leaf herbicides is highly recommended in this region. However, considering the specific environmental conditions of semi-arid regions and the promising potential for expanding camelina cultivation, herbicide-free crop management should

not be overlooked. Mixed cultivation of camelina with crops such as barley or peas, along with increased plant density per unit area and the use of mechanical control methods, has been suggested as an effective approach to improving camelina performance (Leclère *et al.* 2019). This study highlights the importance of irrigation in optimizing camelina performance and maximizing herbicide efficacy. Camelina producers in semi-arid or rainfed regions should consider the use of herbicides like trifluralin, haloxyfop-R-methyl, and pinoxaden under irrigated conditions for better weed control and higher yields. However, under rainfed conditions, producers may need to focus on more integrated approaches for weed management as herbicide effectiveness significantly drops. This further underscores the need for careful irrigation management and herbicide choice to enhance camelina production, particularly in regions where water availability is a limiting factor. Also, population assessment of weed species in the field showed that *Convolvulus arvensis*, *Lactuca virosa*, *Raphanus raphanistrum*, *Sisymbrium officinale*, *Capsella bursa-pastoris*, *Lepidium draba*, *Chenopodium album*, *Portulaca oleracea*, *Bromus tectorum*, *Hordeum jubatum*, and *Alhagi maurorum* had the highest population density and biomass after pesticide application.

This study revealed that certain herbicide and irrigation combinations were less effective in enhancing camelina growth and yield. Specifically, the rainfed treatments (I2) combined with herbicides H1 (no herbicide), H2 (trifluralin), H3 (cycloxydim), H4 (haloxyfop-R-methyl), and H5 (pinoxaden) showed the least favorable outcomes in terms of camelina performance. Among these, the combination of rainfed conditions with no herbicide application (I2-H1) resulted in the highest weed density and biomass, which significantly reduced camelina yield and growth parameters. Similarly, the treatments involving trifluralin (H2) and cycloxydim (H3) under rainfed conditions exhibited weaker performance than the same herbicides under irrigated conditions. These results underscore the necessity for adequate irrigation to optimize the efficacy of herbicide treatments and minimize weed competition, particularly in water-limited environments. The least effective herbicide and irrigation combinations, therefore, include all rainfed treatments, especially when paired with trifluralin, cycloxydim, haloxyfop-R-methyl, and pinoxaden. These combinations were shown to result in lower agronomic traits, including seed yield, number of siliques per plant, and seed number per silique, than treatments under irrigated conditions. While this study provides valuable insights into the interaction between herbicides and irrigation conditions for camelina cultivation, further research is needed to better understand the long-term impacts of different herbicide applications, particularly under rainfed conditions. In particular, research should explore alternative weed management strategies that are more effective in water-scarce environments, as well as the potential development of herbicide resistance in narrow-leaved weed species such as *Avena sterilis* and *Phalaris minor* under continuous use of ACCase-inhibiting herbicides. Additionally, studies investigating the optimization of irrigation techniques, combined with herbicide treatments, would be beneficial for maximizing camelina productivity in semi-arid and drought-prone regions. The potential for alternative herbicides or integrated weed management practices, including crop rotation and mechanical weed control, should also be explored to mitigate herbicide resistance.

Conclusions

Camelina cultivation under irrigated conditions with the application of cycloxydim (H3), haloxyfop-R-methyl (H4), and pinoxaden (H5), led to optimal performance in terms of seed yield, number of siliques per plant, and seed number per silique. These herbicides were found to be highly effective in controlling narrow-leaved weeds, thus reducing competition for water, nutrients, and light, which ultimately benefit the growth and productivity of camelina. In contrast, weed density and biomass were higher in treatments with no herbicide application, highlighting the importance of weed management in ensuring optimal plant growth. It is also important to note that overuse of ACCase-inhibiting herbicides (like cycloxydim, haloxyfop-R--methyl, and pinoxaden) could lead to resistance in certain weed species. To mitigate this, it is recommended to rotate herbicide types or combine them with other weed management strategies to prevent resistance buildup.

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