Journal of Advances in Microbiology

Volume 24, Issue 10, Page 62-75, 2024; Article no.JAMB.122808
ISSN: 2456-7116

Biopesticide Effect of Potential Microorganisms in Bioformulation Against *Phytophthora infestans* (mildew) and *Alternaria* spp. (alternariosis) on *Capsicum annuum* L. in Field

Zoa Parfait Alexis [a*], Effa Onomo Pierre [a], Fotso [b] and Djocgoue Pierre-François [a]

[a] Faculty of Science, Laboratory of Plant Biotechnology and Environment, Biochemistry and Molecular Biology Unit, University of Yaounde I, Yaounde, P. O. Box-817, Cameroon.
[b] Department of Plant Biology, High Teacher's Training College, University of Bamenda, Bambili, Bamenda, P. O. Box-39, Cameroon.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/jamb/2024/v24i10858

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/122808

Original Research Article

Received: 14/07/2024
Accepted: 18/09/2024
Published: 03/10/2024

**Corresponding author: E-mail: zoaparfait69@gmail.com;*

Cite as: *Alexis, Zoa Parfait, Effa Onomo Pierre, Fotso, and Djocgoue Pierre-François. 2024. "Biopesticide Effect of Potential Microorganisms in Bioformulation Against Phytophthora Infestans (mildew) and Alternaria Spp. (alternariosis) on Capsicum Annuum L. In Field". Journal of Advances in Microbiology 24 (10):62-75. https://doi.org/10.9734/jamb/2024/v24i10858.*

ABSTRACT

The production of pepper, a staple food of millions of people in the world is facing a severe parasitic pressure of pests and diseases. The objective of this work aimed at developing a biopesticide formulation (T1) using potential microorganisms as *Trichoderma harzianum* and *Metharizium anisopliae* and test it performance against the virulence of *Alternaria solani* and *Phytophthora infestans* isolated from two pepper varieties V1 (*Safi*) and V2 (*Burkina yellow*) in two agroecological areas. The previous formulation was made up with rice bran fermentation and potential microorganisms specifically selected. The two-factor split-plot design consisted of 06 basic plots per block for each pepper variety. A significant difference ($p = 0.05$) was revealed between the formulation (T1), chemical pesticide as positif control (T2) made up with Mandipropamid and the control (T0) in the field on disease incidence and severity in all stage of pepper growth. Mildew incidence ranged from 12.72% to 53% for the V1 and 10.01% to 49% for the V2 in agro-ecological area III. Similarly, 8.99% to 60.9% for variety V1 and 7.12% to 55% for variety V2 in agro-ecological area V between 4 and 12 weeks. The incidence of alternariosis varied from 7.12% to 77.12% for variety V1 and 7.01% to 69.15% for variety V2 in agro-ecological area III. Also 4.32% to 71.9% for variety V1 and 5.12% to 67.08% for variety V2 in agro-ecological area V between 4 and 12 weeks. Gas chromatography combined with mass spectrometry revealed an overproduction of bioactive molecules in the hydro-methanolic extracts of pepper leaves treated with the formulation T1, which are responsible for its phytoprotection in the two agroecological areas. In conclusion, the use of the *Trichoderma harzianum* and *Metharizium anisopliae* formulation significantly induced systemic resistance of *Capsicum annuum* L plants against alternariosis and mildew agents.

Keywords: Alternariosis, mildew; Trichoderma harzianum; Metharizium anisopliae; Capsicum annuum L; bioprotective molecules.

1. INTRODUCTION

Agriculture is an important economic sector in Cameroon. It contributes 15.6% of the national Gross Domestic Product [1]. However, Cameroon is committing substantial resources to ensuring the success of the agricultural sector, which has not yet made any significant headway due to a major problem linked to the attack of cultivated fields by insect pests and phytopathogens, causing serious damage to economically important crops [2].

Food crops are threatened annually by a wide range of pests, including micro-organisms, animals (vertebrates and invertebrates) and plants (weeds). In order to reduce crop damage, predator control strategies must be put in place. The use of synthetic pesticides has proved effective in protecting crops, but it has also increased the environmental impact in terms of soil and water contamination and phytotoxicity. There have also been a number of cases where these products have lost their efficiency, due to pest resistance [3]. Pepper diseases cause quantitative and qualitative crop damage throughout the world [4]. In Cameroon, the spectrum of occurrence and development of pathologies is increasing over the years, and the literature provides few details of the disease. One reason why farmers find it difficult to implement an effective control strategy [4]. Phytopathogenic fungi are species of parasitic fungi that cause cryptogamic diseases in plants. These fungi belong to the various groups of the oomycete and/or eumycete kingdom ("true fungi"): ascomycetes, basidiomycetes, chytridiomycetes, zygomycetes and deuteromycetes (imperfect fungi) [5]. They are able to infect any tissue at any stage of plant growth, undergoing a complex life cycle that may involve sexual or asexual reproductive stages [6]. Despite its nutritional and economic importance, there is a growing shortage in capsicum production. This is mainly due to the negative impacts of phytopathogenic agents that cause diseases of various levels of severity on this plant. *Alternaria* spp. and mildew are common fungi in our environment. They have saprophytic and phytopathogenic lifestyles that can affect field crops or plant products during harvest and post-harvest [7]. *Alternaria solani,* one of the Alternaria pathovars, is a phytopathogenic fungus of the Pleosporaceae family. It has been reported for several decades as a pathogen of Solanaceae and has long been described as affecting pepper as well as tomato, eggplant, potato, and several species of this botanical family [4]. It causes a disease called

"Alternariosis" or "Alternaria blight" by secreting toxins such as tenuazonic acid, alternariol, AOH-9-glucoside, altenariol monomethylether-3-glucoside [8]. Pepper mildew, on the other hand, is a disease that can destroy entire fields over long distances. It must be regarded as a disease of "collective scope", and it is vital that preventive and curative control measures are adopted for better biocontrol. Under certain conditions*, P. infestans*, the pathogen linked to this infection, can produce survival structures (oospores) that enable it to survive for several years in the soil, without a host plant. However, oospore production is only possible when two reproductive types (type A and type B) are present concurrently in the same field. High relative humidity (>90%) and medium temperatures (between 10 and 15°C at night and 15 and 21°C during the day) are favorable conditions for mildew development. On the other hand, prolonged periods of drought and hot temperatures (> 30°C) are detrimental to its survival and dispersal [9].

Over the past few years, interest in biological protection has been renewed, due to a growing concern for better protection of the environment, and the desire for product quality imposed by consumers. The use of biological control agents has now become a reality in agriculture, particularly for the control of plant pathogens. Biological control using effective bacterial and fungal antagonists is also one of the strategies adopted to manage pests [10]. Indeed, these micro-organisms generally control their target pathogens through various mechanisms, including antibiosis, mycoparasitism, competition for space and nutrients, production of lytic enzymes and secondary metabolites, induction of plant resistance systems and inactivation of pathogen enzymes [11]. Thus, the inhibitory effects of microorganisms on plant pathogens and pests are increasingly being explored [12]. Commercial microbial inoculants (commonly used as biofertilizers or biostimulants) containing single species or strains of Rhizobia, *Pseudomonas* spp, *Azotobacter* spp, *Bacillus* spp, *Trichoderma* spp, *Aspergillus* spp, and *Glomus* spp have been widely used in small agroecosystems for crop production [13]. Taking for example the case of certain *Bacillus subtilis* strains, which are the most extensively studied. Their inhibitory effect has been demonstrated against *Fusarium oxysporum, Sclerotinia sclertiorum*, *Rhizoctonia solani* and *Colletotrichum gloeosporioides* [14]. In addition, *Bacillus subtilis* is a bacterium widely known for its insecticidal effects in agriculture. Hence their phytoprotective effect.

2. MATERIALS ET METHODS

2.1 Study Location

The study was carried out in agro-ecological areas III, IV due to the high trend of pepper cultivation. The study in area III took place in the locality of Njichom in the Noun Division. It is located at altitude 1112 m, with geographical coordinates 5°47'43" N and 10°56'29" E. Its climate is Sudano-Guinean, with an average temperature of about 21.2°C and average annual rainfall of 609 mm (Anonymous, 2019). Area IV is located in Mbele II, in the Obala Subdivision of the Lekie Division. This locality is at an altitude of 528 meters, with geographic coordinates of 4°10'0" N and 11°31'60" E. Its climate is tropical humid, with a relative temperature of 24.7°C and average annual rainfall of 1,638 mm [15].

2.2 Materials

The microbial strains used in the study were *Trichoderma harzianum* (2x10^9 CFU/g) and *Metharisium anisopliae* (10^5 CFU/g), acquired from Dora Agritech laboratories in China. The plant material included two of Cameroon's most valued pepper varieties, *Safi* (Variety V1) and *Burkina yellow* (Variety V2). They have a high yield capacity of about 20 to 40 T/ha. The chemical pesticide Mandipropamid 25%, marketed under the name REVUSTOP or REVUS 250sc, was used as a positive control.

2.3 Morphological Identification and Compatibility Tests between Microorganisms

Trichoderma harzianum and *Metharisium anisopliae* isolates were morphologically identified. A 2 g sporal suspension of pure *Trichoderma* spp. and *Metharisium* spp. cultures was made in 98 ml sterile distilled water. After a series of dilutions (10^10) and plating of 1ml on PDA medium, followed by incubation for 7 days at 25°C in the dark, the germinated conidia were observed under a microscope with objective 40 and magnification X400. The conidia were transplanted with a needle onto PDA medium and incubated for one week at 25°C, with the appearance of green mycelium indicating the presence of *Trichoderma* spp. and *Metharisium* spp. [16].

To determine the additive and synergistic effects of the isolates, compatibility between the two microbial strains was determined by performing a diffusion test on modified agar (or Kirby-Bauer test). A 24-hour-old culture loop grown in LB agar (Luria-Bertani) was transferred to 4 glass test tubes containing 5 ml LB broth and incubated for 24 hours on an incubator shaker (New Brunswick Scientific CO., Inc, Edison, NJ 08817, USA) set at 200 rpm and 30°C. The concentration of *Trichoderma harzianum* suspension was then quantified and adjusted to ~108 CFU/ml. Sterile 8 mm Whatman filter paper discs were aseptically immersed in the *Metharisium anisopliae* medium suspension and air-dried for 30 minutes. *Trichoderma harzianum* strain was dabbed evenly onto the plate using a sterile cotton-tipped applicator. Four discs impregnated with the suspension representing *Metharisium anisopliae* were gently pressed onto the *Trichoderma harzianum*-inoculated agar surfaces at four equidistant positions using sterile forceps. Plates were incubated at 28 ± 2°C and observed over a 72 h period.

2.4 Formulation Process

The "vehicle" used was rice bran according to the modified method of Olivera et al. [17]. This involved a mixture of 1kg of each microbial formulation with 1kg of brown sugar in 8l of non-chlorinated water. The mixture was fermented in a hermetically sealed bucket for 07days. The resulting product was then mixed with 50kg of rice bran and transferred to a drum for further fermentation of 7 days for fast growth of the microorganisms. The portion to be sprayed as a biopesticide was prepared using 5kg of the solid fraction mixed with 5L of molasses and 5L of mildew. This was mixed in a 100L drum, topped up with non-chlorinated water, then stirred and fermented for 7 days. The product was then ready for use in the field.

2.5 Field Preparation and Bioinoculation

The two-factor split-plot design consisted of 06 basic plots per block for each pepper variety, separated from each other by an interval of 1 m and from each other by an interval of 2 m for each pepper variety. Plants were spaced 50 cm apart. All the plots in a block represented the same treatment. Each plot had 20 plants. Every two plots in the blocks consisted respectively of treatments T0 (control); T1 (*Trichoderma harzianum* and *Metharisium anisopliae)* and the positive treatment T2 (Mandipropamid). The biopesticide based on beneficial microorganisms and the chemical pesticide were applied to the crop soil a week before sowing in order to reduce the impact of phytopathogens. Other applications were made once every 7 days until harvest. For the treatment at T3, the application for the same frequencies was based on spraying by mixing 25 mL of Mandipropamid in 10 L of water.

2.6 Symptomatic identification and characterization of *Alternia* spp. and *Phytophthora infestans*

Observations on the leaves of seedlings in the field were made from 4 weeks after sowing to the first harvest corresponding to 10 weeks. Leaf symptoms were observed and described for each accession. Appearance, shape, size and coloration of symptoms were described by visual observation. Leaves and fruits from accessions showing different types of fungal attack symptoms were randomly sampled. Thus, five leaves with the same type of symptoms were collected from five plants per treatment for each variety in the different agro-ecological areas and placed in blotting paper, and then in a polyethylene plastic bag for laboratory analysis.

Colonies of *Alternaria* spp. spores were identified through the symptoms observed on the plant and fruits using infected fruits, which were washed in 200ml of water. This solution was filtered through 0.45µm millispore membranes. The gelatin/glycerine cube was used to recover spores from the filter surface. These were then transferred to PDA culture medium in petri dishes. Culture was carried out in an incubator at 28°C for 7 days, with photoperiods of light and dark for 08 h/16 h. The spore-laden surface was scraped to remove spores using a metal spatula and distilled water. The resulting suspension was filtered through muslin to separate the spores from the mycelium [18]. The nature of mycelia growth, shape of conidia was noted. Numbers of septa were also recorded.

For plants infected with mildew, symptoms on leaves and fruits were sampled from 20 leaves and tomato fruits of each variety in both agroecological zones. Small fragments of infected plant organs (fruit and leave) were surface-disinfected by dipping in 0.5 % (w/v) sodium hypochlorite for 2-5 min and rinsed with sterile water before they were placed onto potato dextrose containing streptomycin, in 90-mm Petri dishes. Height Petri dishes were used, for each plant organ, each with one pepper variety from

each agroecological zone. Subsequently, plates were incubated at 25 °C until the mycelial growth was observed. After 3-5 days of growth on PDA, isolates were identified by morphological and cultural characteristics [19]. Disease severity on plants was evaluated in both aroecological areas on plants cultivars by counting infected plants above 20 plants in each of 6 plots during harvest.

2.7 Resistance of Pepper Plants to Pathogens

The incidence (I) or expansion rate of the disease is the frequency of occurrence of the disease on plants in a block was thus determined and expressed as a percentage according to the formula [20]:

$I = \frac{n}{N} \times 100$

I = Disease incidence
n = Number of infected plants in plot
N = Total number of plants in the plot

Once these two parameters had been calculated, mean values were compared between the treatment and the T0 control.

2.8 Pathogenicity Test

The pathogenicity test was performed on healthy young pepper leaves placed on blotting paper soaked in distilled water in Petri dishes (Ø = 90 mm) and treated with the spore suspension (10 µL). Petri dishes containing 10-day-old pure isolates of both *Phytophthora infestans* and *Alternaria* spp. on PDA medium were selected for the preparation of spore suspensions. Healthy control leaves were treated with sterile distilled water (SDS). Inoculated dishes were sealed and incubated under laboratory conditions at 23°C. Observations were made from day 2 of treatment to day 15 [21].

2.9 Phytochemical Screening

Extracts of leaves treated with combination of *Trichoderma harzianum* and *Metharisium anisopliae* in field and non-treated leaves (control) were analyzed using GC/MS. Leaf extraction was carried out on soxhlet delipidated leaves (with methanol as solvent) according to the method of Oomah et al. [22] by reflux decoction of 50 g of powder in 500 ml of a hydro-methanolic mixture (20V/80V respectively) for 30 min, with the maximum aim of extracting polar compounds such as polyphenols. After filtration on Whatman No.1 paper, the filtrates obtained were evaporated using a rotary evaporator at 60°C. The filtrate residues were oven-dried for 48 h at 45°C to obtain the dry extracts. The methanolic extract was analyzed by capillary gas chromatography combined to mass spectrometry (GC/MS) using an Agilent 7890A GC coupled to an Agilent MSD 5975C inert mass spectrometer. The gas chromatograph was equipped with a VF-1MS capillary column (100% dimethylsiloxane, 20 m x 150 µm (internal diameter) x 0.15 µm (film thickness)) from Varian, liner internal diameter 4 mm. Carrier gas: helium (constant flow rate of 1 mL/min); oven programming: from 37°C (1 min) to 250°C at 5°C/min, then 11 min step-up to 250°C; source temperature: 230°C; transfer line temperature: 260°C; ionization energy: 70 eV. Electron ionization (EI) mass spectra were recorded between 40-400 u. In mass spectrometry, detection is based on the mass-to-charge ratio (m/z) of an analyte. The analyte molecules in the vapor phase are bombarded with a high-energy electron beam. Positive ions are produced by bombardment in a magnetic field on the basis of their m/z ratio. The signal emitted by the ions from the spectrum is recorded and presented as a computer-generated graph. Constituent identification was based on comparison of mass spectra with commercial Wiley and in-house laboratory databases. Retention indices were calculated using the homologous alkane series (C7-C40) and compared with retention indices available in the NIST Webbook after Kondjoyan & Berdague, [23]. Each analysis was performed in triplicate after experimental optimization.

2.10 Statistical Analysis

The results were analysed statistically by the one-way variance analysis method. The Tukey test was used to compare the different means in order to highlight any significant differences between them. Data were first registered on Excel version 2016 and then analyzed using the Rcmd package of Rversion 3.6.3 softwares.

3. RESULTS AND DISCUSSION

3.1 Test of Compatibility

Test was carried out between combinations of the two microbial strains to assess their ability to cohabit in the same bioformulation. It has then been provend that *T. harzianum* and *Metharizium anisopliae* have developed a capacity to cohabit in the same environment. In the process of organic matter management technologies, more

attention has to be paid to microbiology, and physical, chemical and technical approaches to ensure plant protection against phytopathogens while causing more environmental damage. All those factors are supposed to be taken into consideration when producing bioformulations.

Development of an easy applicable biopesticide most therefore take in account the delivery system for the application of the field. Thus, it is important to determine the size of the particle and flow properties which are parameters that can help to predict the distribution and to choose the ingredient that may favor good distribution of the active ingredient [24].

3.2 Symptomatic identification and characterization of *Alternia* spp. And *Phytophthora infestans*

Direct and indirect techniques were used to obtain isolates from mycelium grown on pepper sections and leaves in the laboratory. Macroscopic (Figs. 1&2) and microscopic (Figs. 1B and 1C) characteristics of pure strains from the two agro-ecological areas of pepper production were observed. The pure strains showed short, whitish, aerial mycelial filaments. These mycelial filaments, observed under a light microscope at X100 magnification, are unpartitioned and display terminal sporocysts (Figs. 1B&2B) which differentiate after heat shock into zoospores (Fig. 1C) typical of *P. infestans.*

Beninal [25] highlighted this difficulty when characterizing *Phytophthora infestans* populations in Algeria. This could be explained by the low competitiveness of *P. infestans* with saprophytic fungi. Morphological and morphometric characterization of *P. infestans* isolates showed significant variation in morphological characters. These morphological characteristics are similar to those described by Gallegly & Hong [26] in their work on the morphological identification of Phytophthora species. This can be explained by the fact that *P. infestans* populations are highly diverse in Cameroon. Indeed, Coulibaly et al. [27] had already pointed this out when working on the characterization of *Phytophthora* spp isolates from the cocoa orchard of Côte d'Ivoire. Indeed, the high genetic diversity observed in *P. infestans* populations in Northern Europe and Estonia has been attributed to the involvement of sexual reproduction [28]. The presence of these morphotypes in the two main pepper production basins is thought to result from the different origins of the pepper seeds used in the growing areas [29]. These seeds would be infected by strains of different sexual types.

Morphological characters of *Alternaria* spp. were quite close to those defined by Simmons [30]. Identification of Alternaria relied on morphological and cultural characters which cause identification of *Alternaria* spp. difficult. Purified fungal isolates were identified based on their morphological characters, including spore size, growth pattern and spore chain formation [31]. *Alternaria* spp. is an opportunistic fungus that assumes worldwide distribution and responsible for causing pepper leaf spot disease has not yet been reported in Cameroon. As the morphological characterization of *Alternaria* spp. is a challenging and often misleading task due to the striking similarities among the conidia of several species and the cultural features of some *Alternaria* spp. are unstable [32]. Qin et al. [8] reported that morphological characters used to delineate species in the Alternaria section Alternaria are phenotypically plastic and do not allow the reproducible differentiation of several morphospecies. These characteristics may therefore be misleading in the description and comparison of small-spored catenulate Alternaria isolates in this study [29].

Fig. 1. Macroscopic and microscopic characteristics of pure strains of *Phytophthora infestans* obtained in different agro-ecological areas. A- Pure strain. B- Sporocysts under light microscope; C- Zoospores under photonic microscope (magnification X 100)

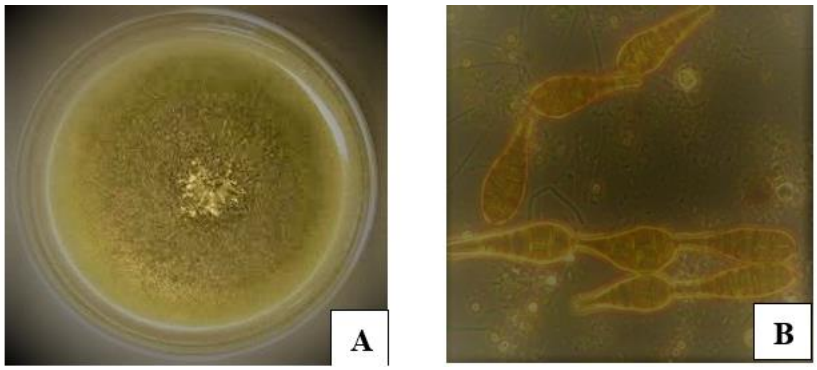

Fig. 2. Macroscopic and microscopic characteristics of pure strains of *Alternaria* spp. obtained in different agro-ecological areas. A- Pure strain. B- Sporocysts seen under a photonic microscope

3.3 Disease Average Incidence and Severity

Surveys carried out in agro-ecological areas III and V, in the localities of Njichom and Mbele II respectively, showed that the incidence and severity of mildew and alternariosis were fairly high in the two pepper varieties studied. All the plots visited in the different localities showed typical symptoms of both diseases. Figs. 3 & 4 show respectively the evolution of the average incidence of mildew and alternariosis in the different localities. Incidences of mildew ranged from 12.72% to 53% for variety V1 (*Safi*) and 10.01% to 49% for variety V2 (*Burkina yellow*) in agro-ecological area III. Similarly, 8.99% to 60.9% for variety V1 and 7.12% to 55% for variety V2 in agro-ecological area V between 4 and 12 weeks. Alternaria incidence ranged from 7.12% to 77.12% for variety V1 (Safi) and 7.01% to 69.15% for variety V2 (Burkina yellow) in agro-ecological area III. Also 4.32% to 71.9% for variety V1 and 5.12% to 67.08% for variety V2 in agro-ecological area V between 4 and 12 weeks. Nevertheless, no significant difference ($P > 0.05$) was observed between the incidences of the different localities for the different periods between the 2 pepper varieties for treatments T1 and T2 but present between these treatments and the T0 control in the above-mentioned agro-ecological areas.

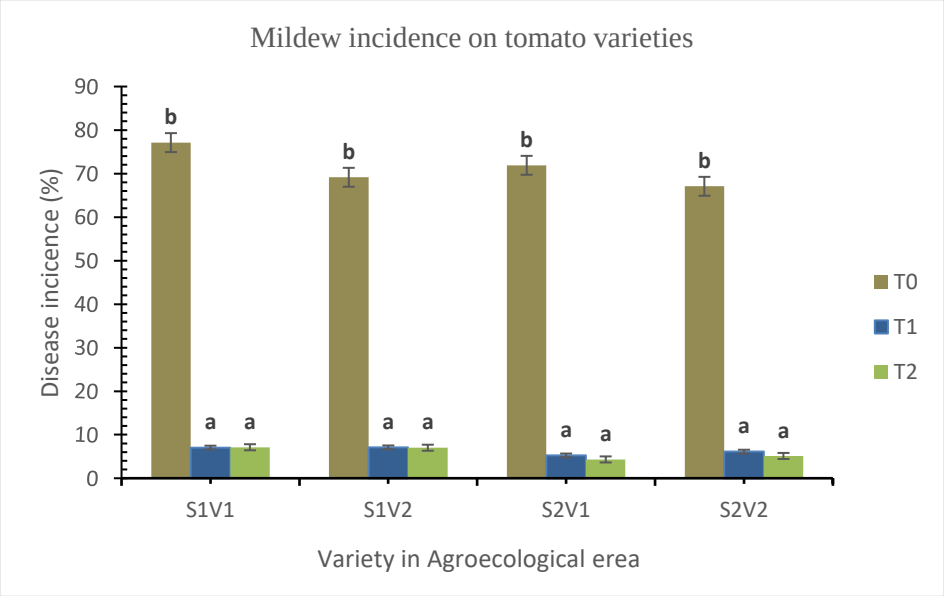

Fig. 3. Evolution of Mildew incidence on pepper varieties in agroecological areas III and V
**Treatments with the same letters at the same zone are not significantly different according to Tukey's test P<0.05*

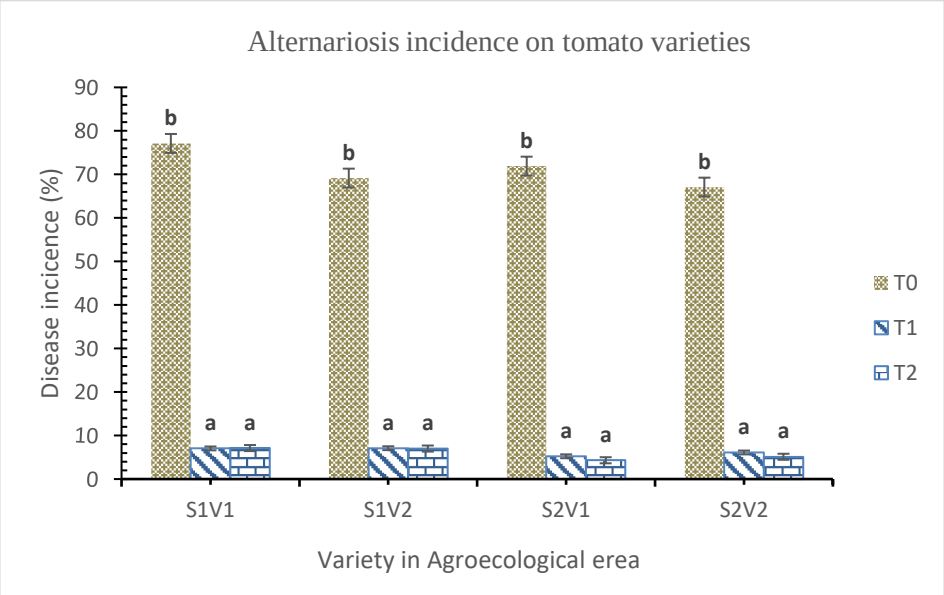

Fig. 4. Evolution of alternariosis incidence on pepper varieties in agroecological areas III and V
**Treatments with the same letters at the same zone are not significantly different according to Tukey's test P<0.05*

The field application of a biopesticide requires an adequate system to spread and deposit the microbial agents. The choice of correct equipment is therefore essential to ensure that the biopesticide is applied effectively at the correct rate and in the target surface. It depends, among other things, on the size of the particles entering the compositions of the final products for example.

Temperature and humidity play a decisive role in the development of mildew, particularly during contamination phases: relative humidity above 90% and average temperatures above 16°C enhance the spread of the disease. In addition, the interaction between climate changes and thermal adaptation of *P. infestans* may have profound effects for the future of pepper. The appearance of the disease in untreated plots was therefore due to the maintenance of ambient temperature conditions favorable to the spread of phytopathogens during the growing season. Incidence and severity were very high in these plots, due to the absence of crop rotation, which accentuates the multiplication of pathogenic fungi [6]. Failure by some growers to comply with certain prophylactic measures, such as crop rotation, to limit primary inoculum [32]. The spatial importance of the crop could also be a factor influencing the importance of the disease in the plots, according to the work of Hammi [33] in the Sais region when he was working on the characterization of *P. infestans* populations. Disease incidence in the two agro-ecological areas ranged from around 49% to 71% for the two pepper varieties with greater susceptibility in zone V. Disease heterogeneity on treated plots is rather the subject of disparities in terms of field spraying frequencies. Based on the results reported here, it could be deduced that virulence of phytopathogens depends on the area where it's been collected. We found that the highly aggressive strains and the most complex races were detected in agroecological area V while weakly aggressive and less complex races were identified in aera III.

The disease cycle of alternaria leaf blight is similar to that of other foliar alternaria diseases. Sources of inoculum include mycelium in overwintering pepper debris, diseased volunteer or wild peppers, and infected seed. In warm temperate areas, overlapping production seasons allow conidia produced in more mature fields to serve as inoculum for nearby more recently planted fields [7]. Conidiophores and conidia are produced at temperatures from 8 to 28°C and in the presence of 96 to 100% relative humidity or free water on the plant surface. Conidia are released and become airborne as the relative humidity drops soon after daylight and winds are above 2 to 3 m/s [5]. In a study of spore dispersal patterns in Ontario, the number of airborne spores detected displayed a circadian periodicity with peak dispersal from 1100 to 1400 h [8]. Spore dispersal increased with decreasing

of relative humidity, transition from wet to dry leaves, and increases in temperature and wind speed. Spore dispersal patterns can then be interrupted by prolonged high winds, long periods of leaf wetness, and cool temperatures [5]. Conidia deposited on leaf surfaces will germinate and infect under appropriate temperature and leaf wetness conditions. At 24°C, damage generally increases with increasing hours of leaf wetness from 8 to 56 h (for example. In addition, infection rate was markedly reduced at night temperatures below 12°C [8]. Pryor et al. [34] reported disease incidence of 63 to 99% on mature plants in a survey of eight fields in the Cuyama Valley of California. In some of the California cases, entire fields were abandoned before harvest due to excessive petiole decay. Estimates of disease incidence from storage houses and markets also reveal the prevalence of black rot.

3.4 Pathogenicity Test

The pathogenicity test of both phytopathogens showed the appearance of specific symptoms similar to those of mildew and alternariosis. Healthy young pepper leaves infected with inocula of both *P. infestans* and *Alternaria spp.* showed symptoms identical to those observed on infected organs in the field, characterized by mycelial growth; necrotic lesions of mildew, and brown spots; sunken lesions of alternariosis on the leaves, among others, similar to those confirmed by Qin et al. [8] and Rekad et al. [29]. The results of pathogenicity test showed that *Metarhizium* spp. were pathogenic on *P. infestans* and *Alternaria spp* in the two agroecological areas. Sharmila and Manjula [35] evaluated the efficacy of formulations made up with *Metharizium* spp. against the larvae of *Spodoptera litura* and *Helicoverpa armigera* (Hubner). They reported 65.35% larval reduction at *S. litura* with talc-based formulation of potential microorganisms. Similar results were obtained in the study of Gurmehar et al. [36] where the maximum larval mortality (63.33%) was recorded. Mortality was also observed to be increased with rising concentration of formulation possibly due to great spore load. The study of Xiaomeng et al. [37] that the conidia of *Metharizium* spp. tended to gather in the depressions and folds of the insect's cuticle. It invaded *S. frugiperda larvae* in various ways, such as direct invasion by germ tubes and hyphae produced by conidia germination, and also invasion by germ tubes forming appressoria and infec tion pegs. *Trichoderma* spp. are filamentous fungi and are found worldwide in soils, on plant roots and are considered competent substrate colonizers and antagonists of other microorganisms, and are therefore used for biological control of plant diseases worldwide [38]. *Trichoderma* spp. may promote plant health by either directly countering pathogens through mycoparasitism, competitive inhibition and antibiosis, or indirectly via enhanced plant growth and stimulating systemic plant defenses [38]. *Trichoderma* spp. showed positive effects on plant growth and suppression of important plant diseases. It has been shown to be effective in controlling Fusarium head blight, Fusarium crown rot and damping off by producing enzymes and secondary metabolites that inhibit the growth and reduce the levels of mycotoxins in wheat grains [39]. Thus, it had positive effects on suppressing leaf diseases such as tan spot disease caused by Pyrenophora tritici-repentis [39], and rus t diseases caused by *Puccinia* spp.

3.5 Phytochemical Screening

Our present laboratory study revealed an overexpression of secondary metabolites in pepper leaves from the treated plot (Table 1), which are specific to treatment T1 compared with the control plot (Table 2). It also demonstrated that the treatments containing fungal conidia (*M.anisopliae* and *T. harzianum*) were effective for the control of *Phythophtora* and *Alternaria* ssp. In general, treatment with *T. harzianum* and *M. anisopliae* resulted in the reduction of plant diseases. Generally, a mixture of plant secondary metabolite with mycopathogen provides eco-friendly stable pest management system compared to environment-deteriorating chemical pesticides. However, the first step to develop a compatible mixture mainly depends on the interaction of these products that might lead to compatible or toxic interaction [40]. It is well-known that both the fungi and plant secondary metabolites demonstrated an entirely different mode of actions to overcome the target host defense mechanisms to cause mortality [41]. Fungal infection evades the host defense mechanism by triggering complex biochemical interactions in the form of series of events through cuticle adhesion, penetration, proliferation, and toxin production, which ultimately lead to the host mortality. On the other hand, plant secondary metabolites are known antagonists that act by interfering with the signaling of the nervous and cellular systems to overcome the target host defense mechanism [42]. We speculate that secondary metabolites

produced by T1 bioformulation like tetradecanoic and/or hexadecenoic acids and biological control effect on these two fungal infections. They also have an inhibitory effect on *Colletotrichum lagenarum*, agent of anthracnose of melon (Cucumis melo), and cucumber (*Cucumis sativus*) [43]. Benhamou & Picard [44] reported a positive correlation between overall plant response and changes in the biochemistry and physiology of plant cells pre-inoculated by *Trichoderma* spp., which were accompanied by structural modifications. Barakat et al*.* [45] reported that *T. harzianum* releases several volatile compounds such as benzene-ethanol, butanoic acid, propanoic acid, palmitoyl chloride, glycerol-1- palmitate and hexadecanoic acid. They have many functions in biological control (butanoic acid, propanoic acid, tetradecanoic acid, benzotriazepine) and as phytoalexins (benzoic acid; scoparone; scopoletin). Abid and Ahmed [41] has shown in previous studies that plant secondary metabolite 1-Chlorooctadecane produced by *Metharizium anisopliae* improved its efficiency EBCL 02049 against date palm dust mites by reducing the killing time and increasing killing capacity as an acaricide. They have also highlighted that fungal spores *M. anisopliae* have virulence levels to kill the infected larvae. The extract of *M. anisopliae* has been characterized to determine the secondary metabolites. It contained a rich source of bioactive chemicals and mycotoxins, responsible for insecticidal activity [46]. *Metarhizium* are known to produce mycotoxins such as destruxins (dtxs), cytochalasins C and D, helvolic acid, myroridins, swainsonine, tyrosine betaine, serinocyclins A and B, aurovertins, hydroxyovalicin, viridoxins, fungerins, metacytofilin, hydroxyfungerins A and B, and 12-hydroxyovalicin and hydroxyfungerin, the 7- desmethyl analogs of fusarin C and (8Z)-fusarin C [47]. In fact, destruxins are most widespread mycotoxin of the secondary metabolites produced by *Metarhizium* [48]. The combination of both beneficial microorganisms revealed biopesticides compounds as Tetrachloroisophthalonitrate zinc ethylenebis (dithiocarbamate), Methyl-2, Benzimidazole carbomate. They have a significant inhibitory effect on the development of Chemical insecticide was less faster than the fungal treatments in reducing infection activity, but less lasting with no significant difference. These differences could be due to the mechanism of action of the different treatments evaluated. *M. anisopliae* could spread in the ant population, whereas *T. harzianum* causes a food shortage by destroying the symbiotic fungus. In contrast, the chemical insecticide killed large number of ants but did not affect the entire population nor did it affect the symbiotic fungus. Systemic resistance to pathogens is therefore induced by these metabolites, which develop allelopathic mechanisms in the plant [46]. It is well-known that both the fungi and plant secondary metabolites demonstrated an entirely different mode of actions to overcome the target host defense mechanisms to cause mortality. Fungal infection evades the host defense mechanism by triggering complex biochemical interactions in the form of series of events through cuticle adhesion, penetration, proliferation, and toxin production, which ultimately lead to the host mortality [49].

Table 1. Specific molecules from the leaves of the two pepper varieties treated with T1 bioformulation

Hypothetical specific molecules to T1	Retention time	Hypothetical formula	Molecular weight	Abundance (%)
7-Hydroxy-3-(1,1-dimethylprop-2-enyl) coumarin	5,51	$C_{14}H_{14}O_3$	230,26	100
5-Chloro-2-pyridinol	6,3	C_5H_4ClNO	129,54	79,08
Tetrachloroisophthalonitrate zinc ethylenebis(dithiocarbamate)	3,98	$C_4H_6N_2S_4Zn$	256,4	210,4
Octadécanoique acid	4,50	$C_{18}H_{36}O_2$	284,47	61,6
Cyclohexylamine, N-(2-chlorocyclopentylidene)-, N-oxide	7,14	$C_{12}H_{13}N_3O_2$	56,10	56,14
Glycerol 1-palmitate	5,64	$C_{19}H_{38}O_4$	330,50	100
1-Chlorooctadecane	6,99	$C_{18}H_{37}Cl$	279,29	98,74
Methyl-2, Benzimidazole carbomate	6,55	$C_8H_8N_2$	298,9	191,19
Palmitoyl chloride	5,64	$C_{16}H_{31}ClO$	274,9	79,39
1H-Imidazole-2-methanol	4,97	$C_{19}H_{23}NO$	98,1	75,14
Tetradecanoic acid	3,99	$C_{14}H_{28}O_2$	228,37	95,76

Table 2. Specific molecules from the leaves of the two pepper varieties with T0

Hypothetical specific molecules to T1	Retention time	Hypothetical formula	Molecular weight	Abundance (%)
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	3,90	$C_{16}H_{24}O_4$	278,3	76,22
Acide Phthalique, 8-chlorooctyl isobutyl ester	3,78	$C_{20}H_{29}ClO_4$	230,3	14,14
Acide Phthalique, hexylpropyl ester	5,84	$C_{22}H_{34}O_4$	362,5	31,12
Diisopropyl adipate	6,30	$C_{12}H_{22}O_4$	230,3	65,27
N-(2-chlorocyclopentylidene) -, N-oxide	7,14	$C_{18}H_{32}O_2$	280,4	53,40

Mandipropamid is a fungicide effective against foliar oomycete pathogens. It is highly effective in preventing spore germination. It is also an inhibitor of mycelial growth and sporulation. Being rapidly adsorbed to the wax layer of the plant surface, mandipropamid provides a rainfast and long lasting barrier to fungal diseases [50]. The study of Rekanović et al. [51] shows that the mandipropamid is highly effective against *P. infestans*, even under high disease pressure, confirming and extending the data obtained in previous trials conducted on potato in Israel [52]. Similar trials carried out on several locations in Serbia, also showed that mandipropamid was the most effective in potato late blight control [51]. Cohen et al. [50] showed in their studies that all the isolates of *P. infestans* were sensitive to mandipropamid, with EC50 values ranging from 0.02 to 2.98 µg/ml. The effects of mandipropamid on Alternaria spp. were the same despite the lack of data in previous work. The results of this study highlight the resistance of pepper induced by the PGPMs of the T1 formulation while T2 (Mandipropamid) effectively exerts chemical control. The cyclohexylamine produced by the plant under the effect of T1 bioformulation is an analogue of mandipropamid [53,54].

4. CONCLUSION

This study aimed at assessing the sensitivity of *Alternaria* spp and *Phytophtora infestans* on pepper using bioformulation with *Trichoderma harzianum* and *Metharizium anisopliae* in field. The results showed that this formulation is highly effective against *P. infestans* and *Alternaria* spp. reducing diseases on the two pepper varieties in both agroecological zones. This bioformulation significantly induce the production of secondary metabolites in the pepper plants revealed by Gas chromatography combined with mass spectrometry which had biopesticide effects. These molecules stimulate the plant's defense mechanisms against agents causing pepper diseases such as Mildew and Alternaria leaf blight. This formulation could thus be useful for plants phytoprotection.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ACKNOWLEDGEMENTS

The authors wish to thank the National Laboratory of Diagnostic Analysis of Agricultural Products and inputs for phytochemical screening and proofreading.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ministry of Agriculture and Rural Development. Agricultural policies around the world: some examples. European service and international. 2023;03:1.
2. Shahid M, Zeyad MT, Syed A, Singh UB, Mohamed A, Bahkali AH, Elgorban AM, Pichtel J. Stress-tolerant endophytic isolate priestia aryabhattai BPR-9 Modulates Physio-Biochemical Mechanisms in Wheat (*Triticum aestivum* L.) for Enhanced Salt Tolerance. International Journal of Environmental Research and Public Health. 2022;19:10883.
3. Martins PMM, Merfa MV, Marco A, Takita MA, Alessandra A. Persistence in Phytopathogenic Bacteria: Do We Know Enough. Weihui Wu, Nankai University, China. 2018;14.
4. Blancard D, Laterrot H, Marchoux G,

Candresse T. A colour Handbook - Tomato Diseases: identification, biology and control. Manson Publishing Ltd. 2012;688.
5. Fritz M, Jakobsen I, Lyngkjær MF, Thordal-Christensen H, Pons-Kühnemann J. Arbuscular mycorrhiza reduces susceptibility of tomato to Alternaria solani. Mycorrhiza. 2006;16:413–419.
6. Garrido C, Fernandez - Acero Francisco J, Carbu M, Gonzalez -Rodriguez Victoria E, Lineiro Eva et Cantoral Jesus M. Molecular microbiology applied to the study of phytopathogenic Fungi. dans Sameh Maghdeldin, Gel Electrophoresis-Advanced Techniques. 2012;139-156.
7. Logrieco A, Moretti A, Solfrizzo M. Alternaria toxins and plant diseases: An overview of origini, occurrence and risks. World Mycotoxin Journal. 2009;2:129-140
8. Qin Q, Fan Y, Jia Q, Duan S, Liu F, Jia B, Wang G, Guo W, Wang C. The potential of alternaria toxins production by A. alternata in Processing Tomatoes. Toxins. 2022;14:827.
9. Shi X, Tian Z, Liu J, Van der Vossen EAG, Xie C. A potato pathogenesis-related protein gene, StPRp27, contributes to race-nonspecific resistance against Phytophthora infestans. Mol Biol Rep. 2012;39:1909–1916
10. Samada LH, Tambunan USF. Biopesticides as promising alternatives to chemical pesticides: A review of their current and future status. OnLine Journal of Biological Sciences. 2020;20:66–76.
11. Mukesh M, Andleeb Z, Dubey MK, Mohd A, Gupta VK, Upadhyay RS. Comparative evaluation of biochemical changes in tomato (Lycopersicon esculentum mill.) infected by Alternaria alternata and its toxic metabolites (TeA, AOH, and AME). Front. Plant Sci. 2016;7:1408.
12. Deguine JP. Biological control: A need for clarification. Agricultural Book. 2023;32:11.
13. Adeyemi NO, Atayese MO, Olubode AA, Akan ME. Effect of commercial arbuscular mycorrhizal fungi inoculant on growth and yield of soybean under controlled and natural field conditions. J. Plant Nutr. 2019;16:85-101.
14. Guillemette T, Bastide F. Trichoderma mushrooms, benefactors for our society. The Conversation. Soulé Adam. 2022. Cah. Agric. 2024, 33.
Available:https://thecon Page 10 de 11 N
15. Endamana D, Sonwa D. Increase productivity in rural areas with high land pressure in the forest zone of Southern Cameroon. Report of activies in Nkometou III. 1998;34.
16. Samuels GJ. Trichoderma: Systematics, the sexual state and ecology. Phytopathology. 2006;96:195-206.
17. Olivera D, Ayala J, Calero A, Santana M, Hernandez A. Agroecological practices in the province Santi Spiritus, Cuba. Effective Microorganisms, Appropriate Technology on Agroecological Bases. 2014;17.
18. Manika S, Swati D, Dinesh SB, Chowdappa P, Selvamani R, Pratibha S. Morphological, cultural, pathogenic and molecular studies of Alternaria brassicae infecting cauliflower and mustard in India. African J. of Microb. Res. 2013;7:3351-3363.
19. Brasier CM, Cooke DEL, Duncan JM, Hansen EM. Multiple new phenotypic taxa from trees and riparian ecosystems in Phytophthora ganapodyides–P. megasperma ITS Clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. Mycol Res. 2003; 107:277–290.
20. Tchumakov AE, Zaharova II. Influence and statistics of disease development. In: Agronome (eds). Disease damage to agricultural crops. Moscou. 1990;5-60.
21. Abdessemed N. Champignons A Potentiel Mycoherbicide (Doctoral Dissertation); 2021.
22. Oomah BD, Caspar F, Malcolmson LJ, Bellido AS. Phenolics and antioxidant activity of lentil and peahulls. Food Research International. 2010;44:436-441.
23. Kondjoyan N, Berdagué JL. A Compilation of relative retention indices for the analysis of aromatic compounds, Edition laboraatory Flaveur, Theix. NIST Webbook Website. 1996;18/06/2013.
Available:
http://webbook.nist.gov/chemistry/les
24. Singh A, Srivastava S, Singh HB. Effect of substrates on growth and shelf life of Trichoderma harzianum and its use in biocontrol of diseases. Bioresource Technology. 2018;98:470-473.
25. Beninal L. Genetic diversity of Phytophthora infestans agent of mildew potato in Algeria. Magister. E.N.S.A. El Harrach. 2011;92.
26. Gallegly MC, Hong C. Phytophthora: Identifying Species by Morphology and DNA Fingerprints. APS Press –The

American Phytopathological Society, St. Paul, Minnesota. 2008;158.
27. Coulibaly K., Kebe B. I, Koffi K. N., Mpika J., Kone D. Characterization of Phytophthora spp isolates of cocoa orchard of Ivory Coast. Journal of Applied Biosciences. 2013; 70: 5567– 5579.
28. Runno-Paurson E., Kiiker R., Joutsjoki T., Hannukkala A. High genotypic diversity found among population of Phytophthora infestans collected in Estonia. Fungal Biology. 2016;120: 385–392. Doi 10.1016/j.funbio.2015.11.008.
29. Rekad Z. F. Phenotypic and genotypic characterization of P.infestans (Mont) de Barry causal agent of potato and tomato mildew in the northwest region of Algeria. Thesis, Algeria, University of Abdelhamid IBN Badis. 2018; 136.
30. Simmons EG. Alternaria themes and variations (244–286) species on Solanaceae. Mycotaxon. 2000;75 :1–115.
31. Rotem J. The genus Alternaria biology, epidemiology, and pathogenicity. APS Press, St. Paul, Minnesota, USA. 1994; 48 :211–215.
32. Montarry J. Adaptive response of populations of Phytophthora infestans, agent of potato mildew, to crop deployment of its host Solanum tuberosum. PhD thesis. 2007; 192 p.
33. Hammi A. Characterization of phytophthora infestans (mont.) De bary populations in the saïs region. National doctoral thesis in biology. Specialization: Phytopathologie. 2003;272.
34. Pryor BM, Davis RM, Gilbertson RL. Detection of soilborne Alternaria radicina and its occurrence in California carrot fields. Plant Dis. 1998;82:891-895.
35. Sharmila T, Manjula K Field evaluation of oil formulations of Nomuraea rileyi (Farlow) Samson against Spodoptera litura and Helicoverpa armigera in groundnut. Int J Plant Protec. 2015;8:142–147.
Available:https://doi.org/10.15740/HAS/IJPP/8.1/142-147.
36. Gurmehar KG, Neelam J, Yadhu Suneja. Pathogenicity of Metarhizium rileyi (Farlow) Kepler, S.A. Rehner and Humber isolates against Spodoptera litura (Fabricius) and their extracellular enzymatic activities. Egyptian Journal of Biological Pest Control. 2021;31:59.
Available:https://doi.org/10.1186/s41938-021-00407-4
37. Xiaomeng Y, Yanli Z, Jincheng Z, Hui D, Xuejing B, Wei L, Zumin G. Pathogenicity, infection process, physiological and biochemical effects of Metarhizium rileyi against Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) larvae. Egyptian Journal of Biological Pest Control. 2024; 34:19.
Available:https://doi.org/10.1186/s41938-024-00781-9
38. Annette P, Peet G, Andreas VT. Pathogenicity of Trichoderma afroharzianum in Cereal Crops. Pathogens. 2023;12:936.
Available:https://doi.org/10.3390/pathogens1207093
39. Ferreira FV, Musumeci MA. Trichoderma as biological control agent: Scope and prospects to improve efficacy. World J. Microbiol. Biotech
40. Gonçalves Diniz A, Barbosa LFS, Da Silva Santos AC, De Oliveira NT, Da Costa AF, Carneiro-Leão MP, Tiago PV. Bio-insecticide effect of isolates of Fusarium caatingaense (Sordariomycetes: Hypocreales) combined to botanical extracts against Dactylopius opuntiae (Hemiptera: Dactylopiidae). Biocontrol Sci. Technol. 2020;30:1–12.
41. Abid H, Ahmed MA. Potential synergy between spores of Metarhizium anisopliae and plant secondary metabolite, 1-Chlorooctadecane for Effective Natural Acaricide Development. Molecules. 2020; 251900.
DOI: 10.3390/molecules2508190
42. Wink M. Plant secondary metabolites modulate insect behavior-steps toward addiction? Front. Physiol. 2018;9:364.
43. Benzohra IE, Bendahmane BS, Youcef BM. Biological control *In vitro* using Trichoderma harzianum et de Trichoderma viride against Ascochyta rabiei, agent of anthracnose of chickpea in Algeria. Algerian Journal of Arid Region. 2016; 13:51-60.
44. Benhamou N, Picard K. Induced resistance: A new plant defense strategy against pathogens. Phytoprotection. 2000; 80:137-168.
45. Barakat I, Chtaina NEL, Guilli M, Ezzahiri B. Efficacy of bacillus amyloliquefaciens and Trichoderma harzianum in the biocontrol of wheat septoria by seed coating. Revue Morroco Review of Plant Protection. 2019;13:9-18.

46. Vyas N, Dua KK, Prakash S. Larvicidal activity of metabolites of Metarhizium anisopliae against Aedes and Culex mosquitoes J Comm Disea. 2015;38:1-3.
47. Dantas TL, Alonso Buriti FC, Florentino ER. Okra (*Abelmoschus esculentus L*.) as a Potential Functional Food Source of Mucilage and Bioactive Compounds with Technological Applications and Health Benefits. Plants. Hussain A. 2021;10:1683.
48. Rizwan-ul-Haq M, AlJabr AM, Al-Ayedh H. Evaluation of host–pathogen interactions for selection of entomopathogenic fungal isolates against *Oligonychus afrasiaticus* (McGregor). BioControl. 2020;65:185–195.
49. Manuel OC, Miguel A, Altamirano-T, Jorge L, Maicelo-Q, Santo T, Leiva-E, Reyna CT, Carlos OC, Lorenzo C, Lizette DMF, Heidel MRV, Mike ACG, Lily JC. Isolation and Characterization of Native Isolates of Metarhizium spp. as a Biocontrol Agent of Hypothenemus hampei in Rodríguez de Mendoza Province—Peru. Agronomy. 2024;14:13-41.
Available:https://doi.org/10.3390/agronomy14071341
50. Yin Y, Miao J, Shao W. Fungicide Resistance: Progress in Understanding Mechanism, Monitoring, and Management. Phytopathology. 2023;113:707–718.
Available:https://doi.org/10.1094/PHYTO-10-22-0370-KD
51. Cohen Y, Rubin E, Hadad T, Gotlieb D, Sierotzki H, Gisi U. Sensitivity of Phytophthora infestans to mandipropamid and the effect of enforced celection pressure in the field. Plant Pathology. 2007;56:836-842.
52. Rekanović E, Stepanović M, Stević M, Milijašević SI, Todorović B. Mandipropamid – a new fungicide for the control of potato blight (Phytophthora infestans). Collection of summaries of the 9th consultation on plant protection, Zlatibor. 2008;78.
53. Park MS, Romanoski CE, Pryor BM. A re-examination of the phylogenetic relationship between the causal agents of carrot black rot, Alternaria radicina and A. carotiincultae. Mycologia. 2008;100(3): 511-527.
54. RB, Wicks TJ. The incidence of *Alternaria radicina* on carrot seeds, seedlings and roots in South Australia. Australas. Plant Pathol. 2003;32:99-104.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/122808