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Eco-Friendly Biocontrol Of Citrus Aphids Using Entomopathogenic Fungi In Algerian Citrus Orchards

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Abstract:
Citrus cultivation constitutes a cornerstone of socio-economic development in the Chlef region of Algeria, supporting both agricultural productivity and rural livelihoods. However, the widespread presence of pesticide residues across environmental compartments has become a pressing global concern, raising critical questions about the long-term sustainability of conventional pest management practices. In response, this study explores the entomophagous potential of selected fungal microorganisms against citrus aphids. Specifically, the pathogenicity of three entomopathogenic fungi Metarhizium anisopliae, Cordyceps militaris, and Fusarium sp. was assessed in vitro against the green citrus aphid (Aphis spiraecola Patch, 1914). Bioassays were conducted in Petri dishes containing citrus leaves treated with fungal suspensions at a concentration of 10^6 spores/ mL. Insect mortality was recorded at two-hour intervals. The results revealed LT_{50} values of 8 hours (larvae) and 27 hours (adults) for M. anisopliae, 17 hours for C. militaris, and 29 hours (larvae) and 35 hours (adults) for Fusarium sp. All fungal isolates induced up to 100% mortality within 36 hours of application.These findings highlight the strong biocontrol potential of the tested fungi against A. spiraecola and support their integration into pest management strategies to reduce chemical pesticide use in citrus orchards both in the Algeria and throughout Mediterranean region.
Key words: *Citrus, biological control, Aphis spiraecola, entomopathogenic fungi, sustainable agriculture, Orchards, Algeria, Mediterranean region.*

1. INTRODUCTION

Citrus cultivation spans the widest geographical range of any fruit crop, thriving across diverse climatic zones thanks to its remarkable adaptability and high economic value. In Algeria, citrus orchards constitute a strategic pillar of the agricultural sector, especially in coastal and subhumid regions where climatic conditions favor intensive production. However, these orchards are increasingly threatened by recurrent and severe pest infestations that significantly impair fruit quality and reduce overall yield (Kazi-Tani et al., 2024; Ouaarous et al., 2025).

Aphids rank among the most destructive pests of citrus crops, causing direct damage through phloem feeding and indirect harm as primary vectors of phytopathogenic viruses, thereby posing a serious threat to plant health and agricultural productivity (Ait Amar et al., 2022; Amokrane et al., 2023; Mathioudakis et al., 2025). As members of one of the most evolutionarily successful insect families, aphids exert considerable pressure on agroecosystems, forest health, and biodiversity. This ecological dominance stems from several traits: prolific reproduction, flexible reproductive modes (cyclical parthenogenesis and sexual reproduction), a polymorphic life cycle (alate and apterous morphs), and an exceptionally broad host range (Csorba et al., 2024).

Among the various aphid species threatening citrus production, *Aphis spiraecola* Patch, 1914 stands out as one of the most destructive and economically significant worldwide (Amokrane et al., 2023; Singh et al., 2024). Chemical control remains the dominant strategy in global pest management programs, despite decades of advocacy for more sustainable alternatives (Deguine et al., 2021). While synthetic insecticides have demonstrated short-term efficacy against a wide range of agricultural pests, their long-term utility is increasingly undermined by escalating resistance, high economic costs, and substantial environmental externalities (Zhou et al., 2024).

The overuse and misapplication of compounds such as phosphine, DDT, organophosphates, and organochlorines have led to the accumulation of persistent toxic residues in agroecosystems; these residues are associated with genotoxic effects including DNA damage in ovarian cells and have been implicated in the rising incidence of neurodegenerative disorders such as Parkinson's disease (Sharma et al., 2019; Shekhar et al., 2024). Moreover, the selective pressure exerted by chemical insecticides accelerates the evolution of resistant pest populations, further complicating control efforts and threatening crop security.

The urgent need for ecologically sustainable and efficacious pest control strategies has directed attention toward microbial biopesticides. These agents present a compelling alternative, offering broad-spectrum activity, minimal risk of resistance development, residue-free application, no pre-harvest restrictions, human safety, and negligible environmental impact (Ayilara et al., 2023; Irsad et al., 2023; Karaoğlan et al., 2024)

Among biopesticide candidates, entomopathogenic fungi stand out as promising agents for regulating pest populations. These microorganisms infect and lethally exploit insect hosts to complete a stage of their life cycle, thereby contributing to natural pest suppression with minimal ecological disruption (Singh et al., 2017; Vivekanandhan et al., 2024). Beyond their insecticidal activity, entomopathogenic fungi engage in beneficial symbiotic interactions that enhance crop health and productivity (González-Pérez et al., 2022), improve soil composition, and modulate the plant microbiome (Peng et al., 2021). Their infection process involves direct cuticular penetration or enzymatic degradation, followed by the secretion of toxic secondary metabolites, an integrated mode of action that reduces the likelihood of resistance development and mitigates the environmental and health risks associated with synthetic chemical pesticides (Emaru et al., 2024).

Building on these ecological advantages, the present study investigates the insecticidal potential of three entomopathogenic fungi: *Metarhizium anisopliae* (Mea), *Cordyceps militaris* (Com), and *Fusarium sp*. (Fus), against both larval and adult stages of *Aphis spiraecola*. The dipping method, endorsed by the FAO for standardized aphid bioassays, enables precise evaluation of fungal toxicity through direct contact exposure.

2. MATERIALS AND METHODS

2.1Animal Material

Citrus leaves naturally infested with *Aphis spiraecola* were collected from an orchard located in the commune of Medjadja, in the northeastern part of Chlef province (Algeria), at an altitude of 152 m. Samples were transported in plastic boxes (20 × 10 × 5 cm) covered with fine-mesh fabric to ensure ventilation and prevent escape. Identification and isolation of *A. spiraecola* larvae and adults were performed under a binocular stereomicroscope using the taxonomic key of Blackman & Eastop (2006). Specimens were maintained under controlled conditions at 26-± 2°C and 40 ± 5% relative humidity until use.

2.2Fungal Material

The fungal strains used in the bioassays were obtained from cultures maintained at the Mycology Laboratory of the National Institute for Plant Protection (INPV), Algiers, Algeria. The strains included *Metarhizium anisopliae*, *Fusarium* sp., and *Cordyceps militaris*, provided as mycelial suspensions in liquid medium. For maintenance, the strains were subcultured on nutrient agar and incubated in the dark at 37°C. Prior to use in bioassays, they were stored at 4°C in slanted nutrient agar tubes (Bouras et al., 2016).

2.3Subculturing and Germination Assessment

Three culture media were prepared for fungal inoculation and subculturing: potato dextrose agar (PDA; Merck, Darmstadt, Germany) for *Fusarium* sp., malt extract agar (MEA) for *Metarhizium anisopliae*, and prickly pear juice agar for *Cordyceps militaris*. The latter was prepared by blending 250 g of fresh prickly pear pads, filtering the juice, and incorporating it with 15 g of nutrient agar. The mixture was stirred until homogenized and autoclaved at 121°C and 1 bar pressure for 30 minutes, following the protocol of Al-Naqeb et al. (2021). Fungal viability was assessed according to Saruhan et al. (2015). Petri dishes were sealed with Parafilm® (American National Can™) and incubated in the dark for 5 to 7 days at 25 ± 1°C. Germination rates were determined microscopically at ×400 magnification after 24 hours of incubation.

2.4Suspension Preparation

To prepare the spore suspensions, 10 mL of sterile distilled water were added to Petri dishes containing sporulated fungal colonies. The surface was gently scraped to release the spores, and the resulting suspension was filtered through a sterile gauze pad to remove residual mycelium and agar fragments. The filtrate was vortexed for 3 minutes to ensure homogenization, then transferred into tilted sterile tubes. Serial decimal dilutions were performed by transferring 1 mL of the homogenized suspension into 9 mL of sterile distilled water, in accordance with the protocol described in (Figure 1). The conidia concentration of the solution was determined using a Malassez cell. For our test, a spore solution with a concentration of 10^6 spores/ml was used. At each dilution, a drop of Tween® 80 was added to prevent spore agglutination and improve their dispersion (Ganassi et al., 2001).

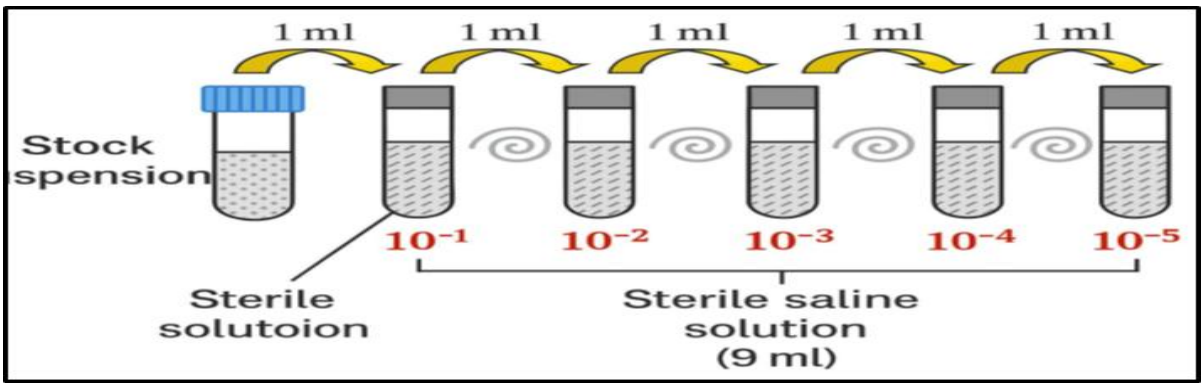

Figure 1: Schematic representation of the serial dilution process used to prepare spore suspensions

2.5Determination of the Insecticidal Activity of the Three Fungi: Contact Test

The insecticidal activity by contact was determined according to the protocol of Ganassi et al. (2001). The experimental unit consisted of 9 cm diameter Petri dishes, the bottom of which was covered with Whatman filter paper (N°. Z146374100EA). A citrus leaf was placed on top to allow for proper insect development during the observation period. The inoculation of aphids (live and parasitized) was carried out by spraying the suspension, using a micropipette, with a 10^6 spores/mL suspension. Each treatment was replicated 3 times. A total of 10 adult aphids and 10 3rd-4th instar larvae were placed separately in each dish. The dishes were covered with perforated plastic film and kept at a temperature of 25 ± 2°C and 40% ± 5% relative humidity. The mortality of the treated individuals was monitored every 2 hours until the death of all individuals. It should be noted that the leaf sections were replaced regularly every two days with new disinfected and rinsed leaf sections. Dead insects were removed before pathogen sporulation to prevent horizontal transmission of the infection within the same dish.

Two controls were included in the experimental design: a negative control consisting of physiological water supplemented with Tween® 80, and a positive control using the synthetic insecticide RUSTILAN® (active ingredient: acetamiprid, applied at 20 mL per 100 mL of water), which served as the reference treatment.

Corrected mortality (CM) was calculated for each replicate using Abbott's formula (1925), which accounts for natural mortality observed in the control group. The formula is expressed as follows:

$$MC = \frac{(M2 - M1)}{100 - M1} \times 100$$

MC: Corrected mortality percentage, adjusted for natural mortality observed in the control group (%).

M_2: observed mortality rate in the treated group (%).

M_1: Observed mortality rate in the negative control group (%).

2.6Statistical Analysis

All statistical analyses were performed using R software (version 4.2.3) within the RStudio environment. A two-way analysis of variance (ANOVA) was conducted to assess the effects of treatment and time, followed by Tukey's Honest Significant Difference (HSD) post hoc test for pairwise comparisons. Results are expressed as mean values ± standard deviation (SD). Statistical significance was established at ($P < 0.05$).

Lethal time estimates corresponding to 50% mortality (LT_{50}) values were estimated using a probit regression model fitted with the glm() function (binomial family, probit link). The proportion of mortality over time was modeled using binary response data (dead/alive). LT_{50} values were extracted using the dose.p() function from the MASS package, corresponding to the time required to reach 50% mortality

3. RESULTS AND DISCUSSION

3.1Macroscopic and Microscopic Characteristics of the Three Entomopathogenic Fungi

3.1.1 Macroscopic Characteristics

Subculturing results revealed distinct differences in the germination behavior of the three-entomopathogenic fungi when grown on their respective media (PDA, JR, MEA). After 10 days of incubation, all three fungi had successfully colonized the media. Notably, *Cordyceps militaris* exhibited early sporulation, visible within 3 days, whereas *Fusarium sp*. and *Metarhizium anisopliae* required 10 days to initiate spore production.

All Microscopic photos were established from the Scientific and Technical Research Center in Physico-Chemical Analysis, located in Setif, Algeria.

Fusarium sp: Colonies *of Fusarium* sp. demonstrated rapid growth on PDA medium. Ten days post-inoculation, the Petri dishes were fully covered by dense fungal colonies, as illustrated in Figure 2. This extensive coverage reflects the vigorous mycelial expansion characteristic of the genus under optimal conditions.

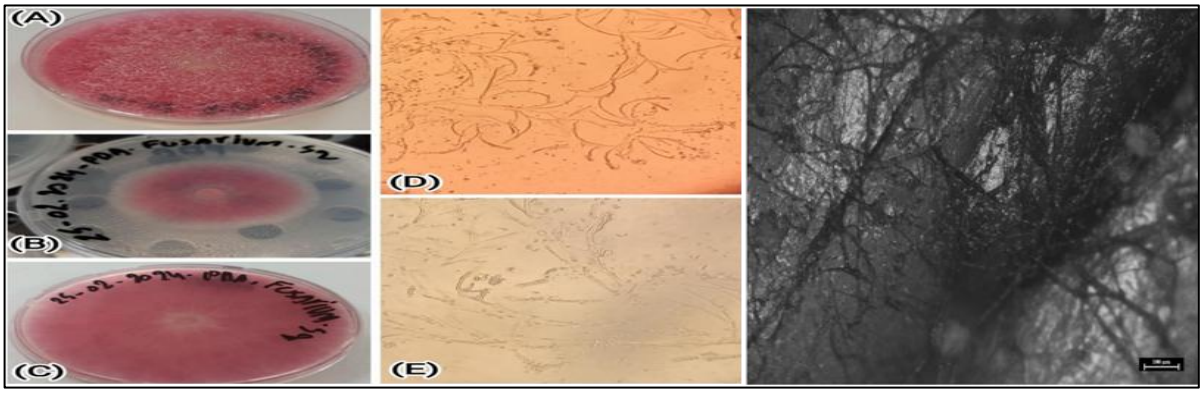

Figure 2: Macroscopic and microscopic characteristics of *Fusarium sp.* A, B, C: Cultural characteristics of *Fusarium sp*. observed on the obverse and reverse sides, respectively. D, E: Mycelia and conidia of *Fusarium sp*. under a microscope at X 10 (D) and X 40 (E)

Metarhizium anisopliae: Macroscopic observations revealed that *Metarhizium anisopliae* exhibited optimal growth on MEA medium, which proved highly conducive to its development Figure 3. After 10 days of incubation, the fungus had fully colonized the medium, forming dense, cottony colonies. During the initial growth phase, these colonies appeared as compact white spheres, gradually expanding into a uniform mycelial mat.

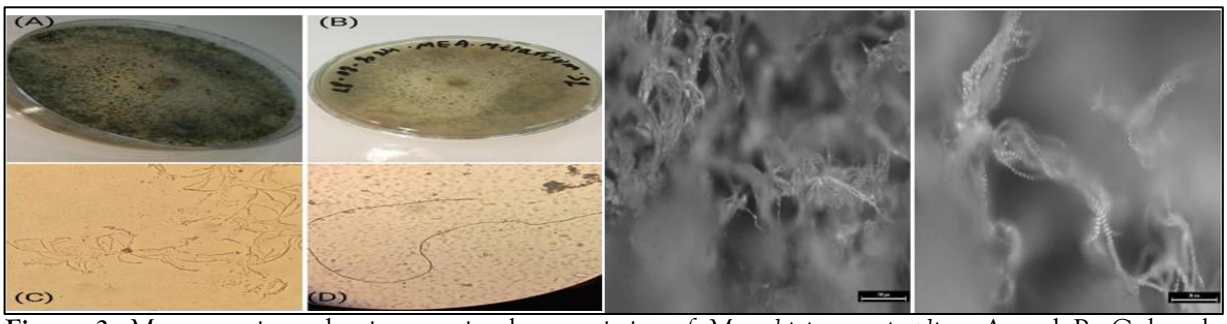

Figure 3: Macroscopic and microscopic characteristics of *Metarhizium anisopliae*. A and B: Cultural characteristics of *Metarhizium anisopliae*, observed on the obverse and reverse sides, respectively. C and D: Conidia and mycelium of *Metarhizium anisopliae*, observed under a microscope (X10 (C), X40 (D), 50 µm, 100 µm)

Cordyceps militaris: Among the three tested media (PDA, JR, MEA), *Cordyceps militaris* demonstrated optimal sporulation on the "racket juice" (JR) medium. Complete colonization of the Petri dishes was observed within just 3 days of inoculation Figure 4, indicating a strong affinity for this substrate. In contrast, growth on PDA and MEA media was markedly slower, with little to no visible development, unlike *Fusarium sp*. and *Metarhizium anisopliae*, which thrived on those respective media.

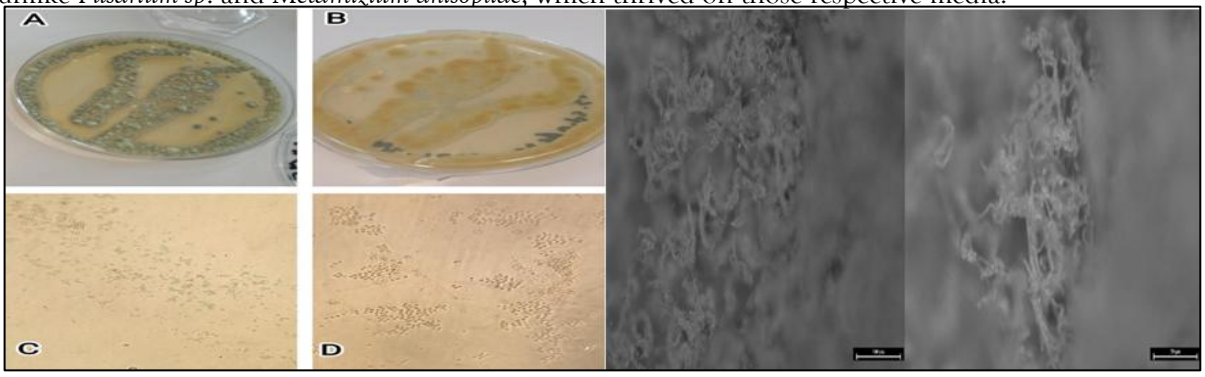

Figure 4: Macroscopic and Microscopic Characteristics of *Cordyceps militaris*. A, B: Cultural characteristics of *Cordyceps militaris* observed on the front and back, respectively. C and D: Cordyceps militaris conidia and mycelium, observed under a microscope (X10 (C), X40 (D), X 50 µm, 100 µm)

3.1.2 Microscopic Characteristics

Microscopic examination of the three-entomopathogenic fungi cultured on PDA, JR, and MEA media revealed distinct morphological traits and medium-specific preferences for each species.

Fusarium sp.: exhibited vigorous growth on PDA medium, forming colonies with a pink, cottony center and white margins. Near the inoculation site, the mycelium was notably denser, giving the colony a flattened conical appearance when viewed laterally. On the reverse side of the Petri dish, concentric rings

were visible, alternating between light and dark pink hues. Microscopic analysis revealed septate, branched, and hyaline (colorless) hyphae, characteristic of the genus.

Metarhizium anisopliae: Colonies of *Metarhizium anisopliae* developed predominantly on MEA medium, displaying a yellow-green base covered by a fine green powder, indicative of the fungus's fruiting structures. The underside of the colonies mirrored this yellow-green pigmentation. Under microscopic observation, the mycelium consisted of hyaline, septate, and branched hyphae. The conidia were smooth, hyaline, and cylindrical, reflecting typical morphological features of the species.

Cordyceps militaris: colonies presented a yellow-green base with cottony, green margins, particularly evident on JR medium. At 40× magnification, the spores appeared variable in size and exhibited a velvety texture. They were hyaline, thick-walled, guttulate, subspherical, and non-amyloid. The mycelium displayed diverse hyphal structures, contributing to the species complex morphology.

3.2. Pathogenicity Test of Different Fungi on *Aphis spiraecola*

A toxic effect was observed for all three entomopathogenic fungal species tested, with mortality rates varying according to exposure duration. The viability of both larval and adult stages of the target pest declined progressively in response to the fungal treatments, indicating a time-dependent pathogenicity. Each fungus demonstrated a distinct level of virulence, reflected in the rate and extent of mortality observed.

The results are presented below, including comparative analyses with both positive and negative controls, to highlight the efficacy of each fungal strain under standardized conditions.

In the present study, we evaluated the pathogenicity of three entomopathogenic fungal species *Metarhizium anisopliae*, *Cordyceps militaris*, and *Fusarium sp*. on both larval and adult stages of the citrus green aphid, *Aphis spiraecola*. Taxonomic identification of the fungal isolates was confirmed through macroscopic and microscopic comparisons with previously characterized species (Benserradj et al., 2014; Nébié et al., 2022; Abdullah et al., 2023).

3.2.1 Effect of Fungi on Larvae *Aphis spiraecola*

The larvicidal activity of the three-entomopathogenic fungi, along with the positive control (RUSTULAN), was evaluated over time against *Aphis spiraecola* larvae. The results demonstrated the pest's clear sensitivity to the biological treatments applied. Progressive mortality was observed, indicating a time-dependent toxic effect for each fungal species.

As illustrated in (Figure 5), the fungal strains induced varying degrees of larval mortality, with *Cordyceps militaris*, *Fusarium sp.*, and *Metarhizium anisopliae* each exhibiting distinct pathogenic profiles. The positive control (RUSTULAN) consistently produced the highest mortality rates, validating the experimental setup. These findings confirm the potential of the tested fungi as biological control agents against *Aphis spiraecola*

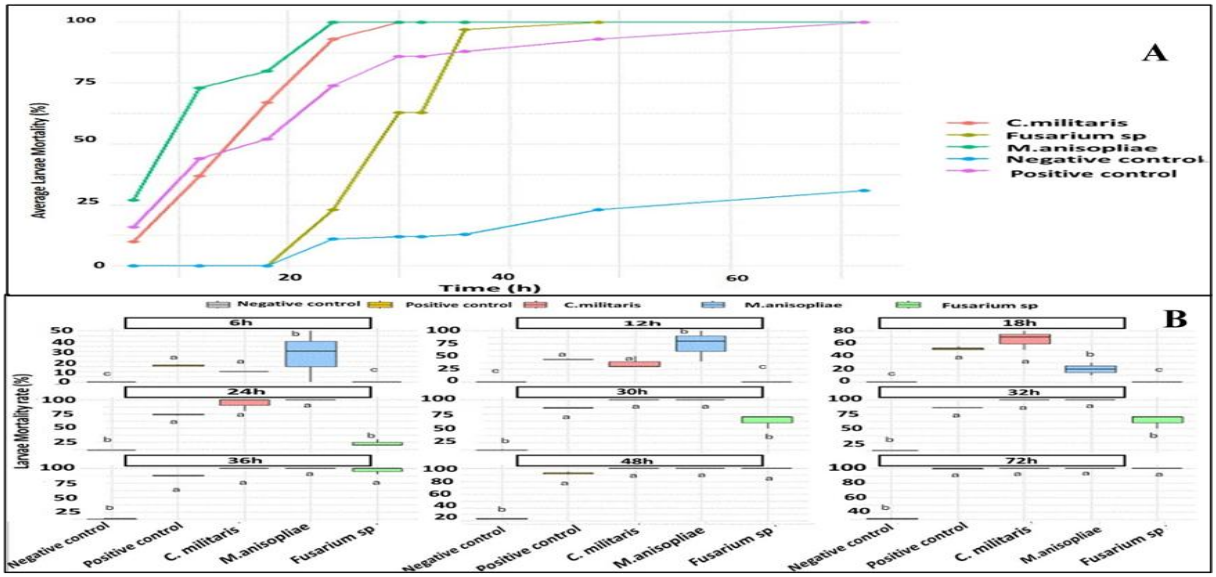

Figure 5. Larvicidal Activity of Three Entomopathogenic Fungi Against *Aphis spiraecola* . A: Kinetics of mortality rates, B: Boxplots of aphid mortality rates

During the initial hours post-treatment (2h to 6h), larval mortality remained low across all conditions, not exceeding 10%. Statistical analysis confirmed no significant differences between the three fungal treatments and the two controls, with all groups classified under the same statistical category (group a).From 6 hours onward, divergence in efficacy began to emerge. By 18 hours post-application, highly significant differences were observed. *Metarhizium anisopliae* (Mea) exhibited the highest average mortality rate (80 ± 0.012%, group b), followed by *Cordyceps militaris* (Com) and the positive control (RUSTULAN),

with respective averages of 67 ± 0.12% and 52 ± 0.02% (both in group a). *Fusarium sp*. (Fus) and the negative control showed no larvicidal activity at this stage.

At 24 hours, the toxic effects of the fungal suspensions became clearly distinguishable. Statistical analysis revealed a highly significant difference between the fungal treatments and the controls. *Metarhizium anisopliae* achieved complete mortality (100 ± 0.00%, group a), followed by *Cordyceps militaris* with 93 ± 0. 09% (group a). In contrast, *Fusarium sp*. showed limited efficacy, with an average mortality of only 23 ± 0.05% (group b), significantly lower than the positive control (74 ± 0.01%, group a).

The time required to reach 100% larval mortality varied by treatment: *Metarhizium anisopliae*: 24 hours; *Cordyceps militaris*: 30 hours; *Fusarium sp*.: 48 hours; Positive control : 72 hours

These results demonstrate that the fungal suspensions of *Metarhizium anisopliae* and *Cordyceps militaris* were more effective than the synthetic chemical product typically used by farmers. Notably, *Metarhizium anisopliae* achieved 100% mortality within 24 hours, while *Cordyceps militaris* reached the same threshold at 30 hours. In contrast, the positive control recorded only 75% mortality at the 24-hour mark. The negative control (physiological water) resulted in no mortality throughout the experiment.

Statistical analysis performed in RStudio on the two-factor treatment (fungus × time) revealed the following groupings: 2h to 18h: Group a: *Metarhizium anisopliae*, positive control Group b: *Cordyceps militaris* Group c: *Fusarium* sp. 24h: Group a: *Metarhizium anisopliae*, *Cordyceps militaris*, positive control Group b: *Fusarium sp*.36h to 72h: All treatments reached 100% efficacy and were classified in group a

ANOVA results (Table 1) provide robust evidence of treatment efficacy over time, with highly significant p-values ($p < 0.0004$ at 24 h, $p < 0.0001$ at 36 h, and $p < 0.0001$ at 48 h). These findings confirm a pronounced dose and time-dependent effect, highlighting the progressive impact of the treatment across the evaluated intervals.

Table 1: statistical significance of the treatment applied to larvae

	6h	12h	18h	24h	30h	32h	36h	48h	72h
C. militaris	10±0,00a	37±0,09a	67±0,12a	93±0,09a	100±0,00a	100±0,00a	100±0,00a	100±0,00a	100±0,00a
M. anisopliae	27±0,21b	73±0,25b	80±0.012b	100±0,00a	100±0,00a	100±0,00a	100±0,00a	100±0,00a	100±0,00a
Fusarium sp	0±0,00c	0±0,00c	0±0,00c	23±0,05b	63±0,09b	63±0,09b	97±0,05a	100±0,00a	100±0,00a
Positive control	16±0,01a	44±0,01a	52±0,02a	74±0,01a	86±0,01a	86±0,00a	88±0,01a	93±0,00a	100±0,00a
Negative control	0±0,00c	0±0,00c	0±0,00c	11±0,00b	12±0,00b	12±0,00b	13±0,00b	23±0,00b	31±0,00b
P > F	0.00065	0.00034	<.0001	0.0004	0.0002	0.00068	<0.0001	<0.0001	<0.0001

3.2.2 Effect of Fungi on Adult *Aphis spiraecola*

The impact of the three-entomopathogenic fungi and the control treatments on adult *Aphis spiraecola* was evaluated under identical experimental conditions. The results, illustrated in Figure 6, reveal distinct mortality patterns over time, highlighting the differential virulence of each fungal strain against the adult stage of the pest.

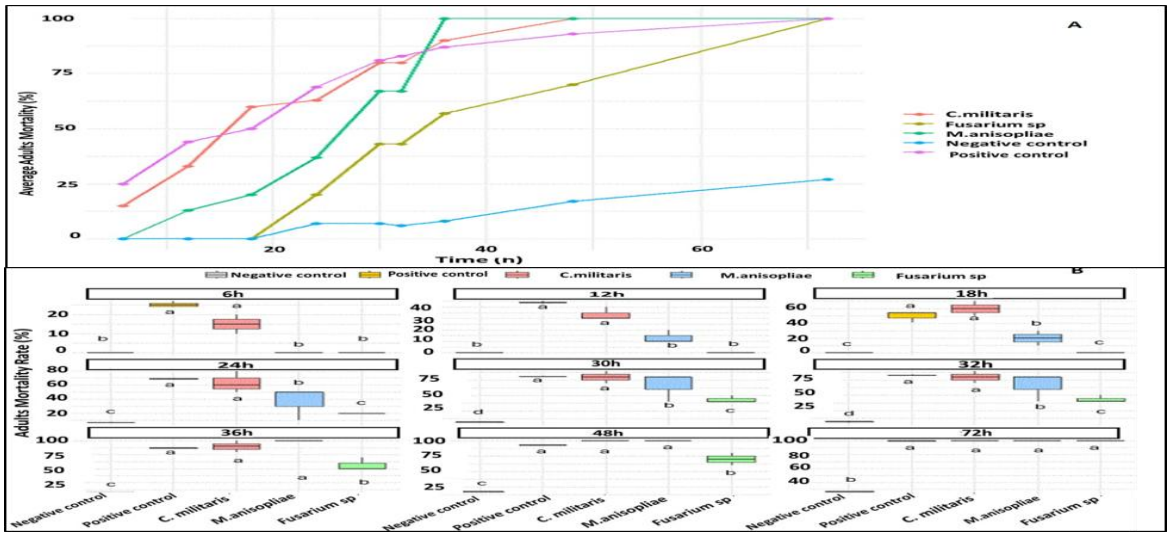

Figure 6. Adulticidal Activity of Three Entomopathogenic Fungi against *Aphis spiraecola* A: Kinetics of mortality rates, B: Boxplots of aphid mortality rates

The adulticidal activity of the three entomopathogenic fungi revealed distinct temporal dynamics. *Cordyceps militaris* (Com) demonstrated the earliest effect, with a mortality rate of 10 ± 0.03% observed just 2 hours after application. In contrast, *Metarhizium anisopliae* (Mea) and *Fusarium sp*. (Fus) reached comparable mortality levels only after 8 and 22 hours, respectively.

Significant differences in adult mortality emerged after 6 hours of treatment. At this time point, *Cordyceps militaris* recorded an average mortality of 15 ± 0.04% (group a), while *Metarhizium anisopliae* and *Fusarium* sp. showed no effect (0 ± 0.00%, group b). The positive control exhibited a mortality rate of 25 ± 0.01% (group a), confirming the onset of toxic activity.

After 24 hours, *Cordyceps militaris* proved to be the most virulent fungus against adult aphids, with an average mortality of 63 ± 0.12% (group a), followed by *Metarhizium anisopliae* (37 ± 0.19%, group b) and *Fusarium sp*. (20 ± 0.00%, group c). Statistical analysis revealed a highly significant difference among treatments, classifying them into three distinct efficacy groups.

Compared to larval mortality, adult mortality was delayed. Complete mortality (100%) was achieved at 36 hours for *Cordyceps militaris*, and slightly later for the other fungi. At 48 hours, *Cordyceps militaris* and *Metarhizium anisopliae* both reached 100% mortality and were grouped together (group a), while *Fusarium sp*. showed a lower efficacy (70 ± 0.08%, group b). The positive control recorded 93 ± 0.01% mortality and was also classified in group (a).

These results underscore the high efficacy of the biological suspensions, particularly those based on *Cordyceps militaris* and *Metarhizium anisopliae*. Notably, their toxic effects were comparable to or even exceeded that of the synthetic chemical insecticide used as the positive control. As illustrated in Figure 6A, the effect of the chemical product plateaued after 32 hours, whereas the biological suspension of *Metarhizium anisopliae* continued to intensify, ultimately leading to complete adult mortality.

ANOVA results (Table 2) provide robust evidence of treatment efficacy over time, with highly significant P values ($p < 0.0006$ at 24 h, $p < 0.0002$ at 36 h and $p < 0.0001$ at 48 h). These findings confirm a pronounced dose- and time-dependent effect, highlighting the progressive impact of the treatment across the evaluated intervals.

Table 2: statistical significance of the treatment applied to adults

	6h	12h	18h	24h	30h	32h	36h	48h	72h
C. militaris)	15±0.04a	33±0.05a	60±0.08a	63±0.12a	80±0.08a	80±0.08a	90±0.08a	100±0.00a	100±0.00a
M. anisopliae	0±0.00b	13±0.05b	20±0.08b	37±0.19b	67±0.19b	67±0.19b	100±0.00a	100±0.00a	100±0.00a
Fusarium sp	0±0.00b	0±0.00b	0±0.00c	20±0.00c	43±0.05c	43±0.05c	57±0.09b	70±0.08b	100±0.00a
Positive control	25±0.01a	44±0.01a	50±0.06a	69±0.01a	81±0.01a	83±0.01a	87±0.00a	93±0.01a	100±0.00a
Negative control	0±0.00b	0±0.00b	0±0.00c	7±0.00c	7±0.00d	6±0.01d	8±0.00c	17±0.01c	27±0.01b
P> F	0.0003*	0.00012	<.0001	0.0006	0.00051	0.00021	0.0002	<0.0001	<0.0001

The corrected mortality rates (MC%) of the tested biocidal treatments were calculated using Abbott's formula (1925), which adjusts observed mortality relative to the negative control to account for natural death rates. These values provide a more accurate assessment of the true toxicological impact of each fungal suspension and the chemical control.

The temporal evolution of corrected mortality is illustrated in (Figure 07), highlighting the comparative efficacy of *Cordyceps militaris*, *Metarhizium anisopliae*, *Fusarium* sp., and the positive control against *Aphis spiraecola*.

Entomopathogenic fungi are capable of infecting a wide range of insect hosts, including both hard- and soft-bodied species, as well as various arthropods (Sharma et al., 2023). Their effectiveness is particularly notable against piercing-sucking insects such as aphids (Vega et al., 2009; Halder et al., 2013). Unlike conventional microbial agents (viruses, bacteria, and nematodes), these fungi penetrate directly through the insect cuticle, bypassing the need for specialized entry routes (Xie et al., 2025).

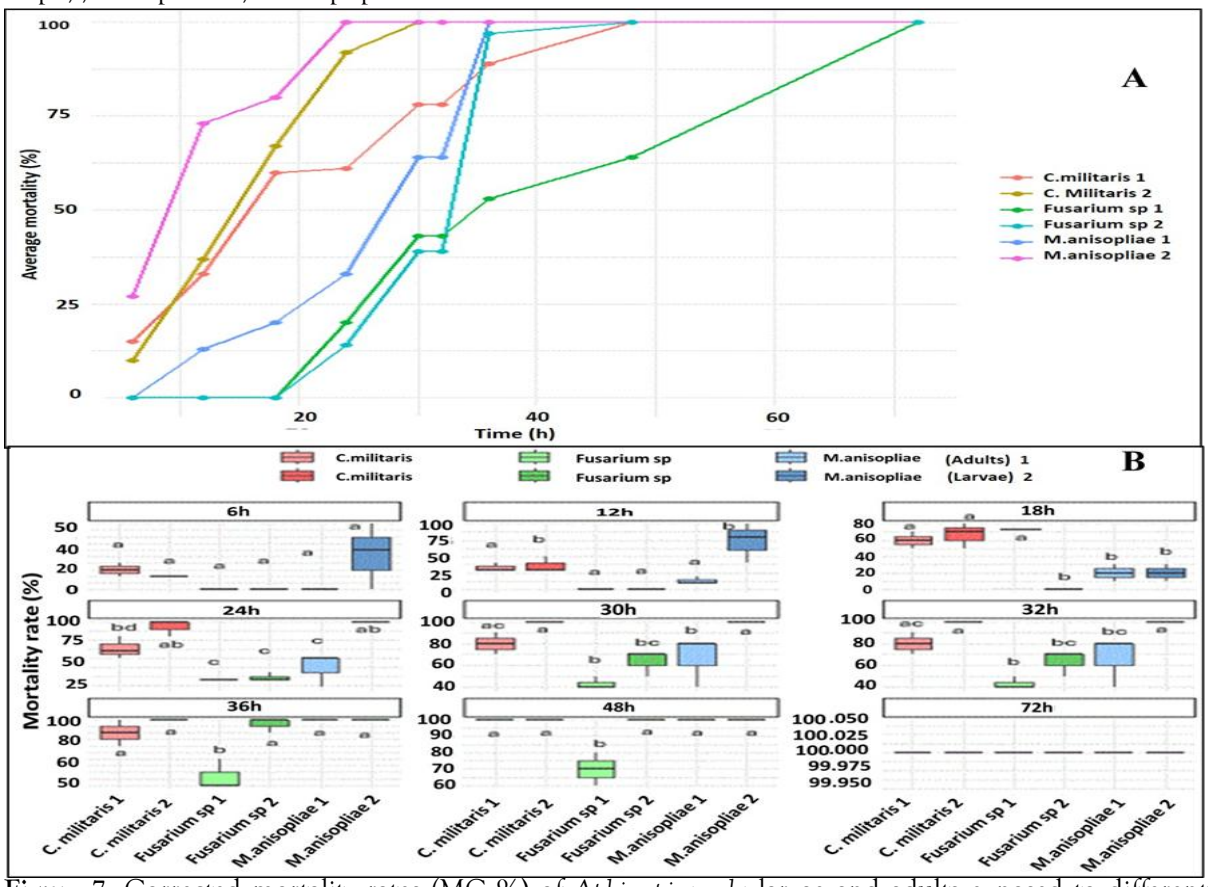

Figure 7. Corrected mortality rates (MC %) of *Aphis spiraecola* larvae and adults exposed to different entomopathogenic fungi under *in vitro* conditions. A: Kinetics of mortality rates, B: Boxplots of aphid mortality rates, 1: Adults, 2: larvae

Additional studies have demonstrated the efficacy of *Metarhizium anisopliae* against other aphid species, including *Brevicoryne brassicae*, *Lipaphis pseudobrassicae*, and *Aphis gossypii* (Jaron *et al.*, 2010; Saldarriaga Ausique *et al.*, 2017; Yun *et al.*, 2017; Hussien *et al.*, 2021). Its ease of cultivation and low susceptibility to opportunistic contamination further support its suitability for commercial biocontrol formulations (Mantzoukas *et al.*, 2022; Emaru *et al.*, 2024). Under natural conditions, aphid colonies are frequently decimated by entomopathogenic fungi, which represent a primary mortality factor (Jaber *et al.*, 2017; Litwin *et al.*, 2020). Infection may occur regardless of the insect's feeding behavior and is influenced by spore dosage, host physiology, and abiotic factors (Vega *et al.*, 2009).

Certain entomopathogenic fungi such as Metarhizium and Beauveria establish endophytic relationships with plants, colonizing roots, stems, and leaves. These interactions can enhance plant growth and nutrient acquisition by parasitizing insect pests (Mweke et al., 2019; Litwin et al., 2020; Aravinthraju et al., 2024; Panwar et al., 2024). Such dual functionality positions these fungi as ecologically sound, cost-effective, and sustainable tools for integrated pest management

3.2.3 Comparative Efficacy and Symptomatology of Entomopathogenic Fungi on *Aphis spiraecola*

Corrected mortality rates, calculated using Abbott's formula (1925), revealed minimal variation due to the low mortality observed in the negative control (≤ 20%). Nonetheless, the biocidal treatments demonstrated distinct temporal efficacy. Larvae treated with *Metarhizium anisopliae*, *Cordyceps militaris*, and *Fusarium sp.* reached 90% corrected mortality within 23.25, and 38 hours, respectively. For adults, the same threshold was achieved at 32.37, and 72 hours, respectively. Based on these results, *Cordyceps militaris* ranks second in efficacy after *Metarhizium anisopliae*, followed by *Fusarium sp.*

These findings align with the Agroscope Arbo (2022–2023) Guide, which emphasizes the heightened sensitivity of larval stages. Delayed treatment may reduce product efficacy and increase larval deposition per flower, underscoring the importance of timely intervention.

To statistically validate these observations, a two-factor analysis was conducted in RStudio to assess the effects of fungal species and aphid developmental stage on mortality rates (Figure 07). During the first 6 hours post-treatment, no significant differences were observed. However, from 8 hours onward, *Cordyceps militaris* and *Metarhizium anisopliae* formed two distinct efficacy groups: Group (a) – Adults: *Cordyceps militaris* (33 ± 0.05%), *Metarhizium anisopliae*:(13 ± 0.05%), Group (b) – Larvae: *Cordyceps militaris* (37 ± 0.09%), *Metarhizium anisopliae*:(73 ± 0.25%)

Fusarium sp. exhibited a mortality rate of (20 ± 0.00%) within 24 hours, reaching 100% only after 72 hours. In contrast, *Metarhizium anisopliae* and *Cordyceps militaris* achieved complete mortality by 38 hours.

ANOVA results (Table 3) provide robust evidence of treatment efficacy over time, with highly significant P values ($P < 8^{e}$-6 at 24h, $P < 2.12^{e}$-5 at 36h, and $P < 3.89$e-6 at 48h). These findings confirm a pronounced dose- and time-dependent effect, highlighting the progressive impact of the treatment across the evaluated intervals.

Our results confirm that fungal suspensions are highly effective in controlling *Aphis spiraecola*, a major citrus pest. These findings are consistent with those of Nébié et al. (2022), who also reported superior efficacy of *Metarhizium anisopliae* and *Cordyceps militaris*, followed by *Fusarium* sp. The reduced virulence of *Fusarium sp.* may be attributed to its mechanical mode of action, which appears less suited to aphid anatomy.

Table 3: Mortality of larvae and adults in different groups over time

	6h	12h	18h	24h	30h	32h	36h	48h	72h
C. militaris (1)	15±0.04a	33±0.05a	60±0.08a	63±0.12bd	80±0.08ac	80±0.08ac	90±0.08a	100±0.00a	100±0.00
C. militaris (2)	10±0.00a	37±0.09ab	67±0.12a	93±0.09ab	100±0.00a	100±0.00a	100±0.00a	100±0.00a	100±0.00
M.anisopliae (1)	0±0.00a	13±0.05a	20±0.08b	37±0.19cd	67±0.19bc	67±0.19bc	100±0.00a	100±0.00a	100±0.00
M. anisopliae(2)	27±0.21a	73±0.25b	80±0.012a	100±0.00a	100±0.00a	100±0.00a	100±0.00a	100±0.00a	100±0.00
Fusarium sp (1)	0±0.00a	0±0.00a	0±0.00b	20±0.00c	43±0.05b	43±0.05b	57±0.09b	70±0.08b	100±0.00
Fusarium sp (2)	0±0.00a	0±0.00a	0±0.00b	23±0.05c	63±0.09bc	63±0.09bc	97±0.05a	100±0.00a	100±0.00
P> F	0.445 ns	0.0002	2.93^{e}-6	8^{e}-6	0.0005	0.0003	2.12^{e}-5	3.89e-6	0.458

3.3 Symptomatology of Fungal Infection in *Aphis spiraecola*

Post-infection symptoms varied by fungal species, though body discoloration was a consistent indicator of mortality (Figure 08).

Metarhizium anisopliae: Dead aphids were initially covered in dense white mycelium, which transitioned to dark green after 48 hours.

Fusarium sp: Induced rapid disintegration of aphid bodies, accompanied by sparse white mycelium and a distinctive pinkish hue.

Cordyceps militaris: Exhibited endoparasitic behavior, consuming the host internally before emerging through the head to release airborne spores, facilitating further infection

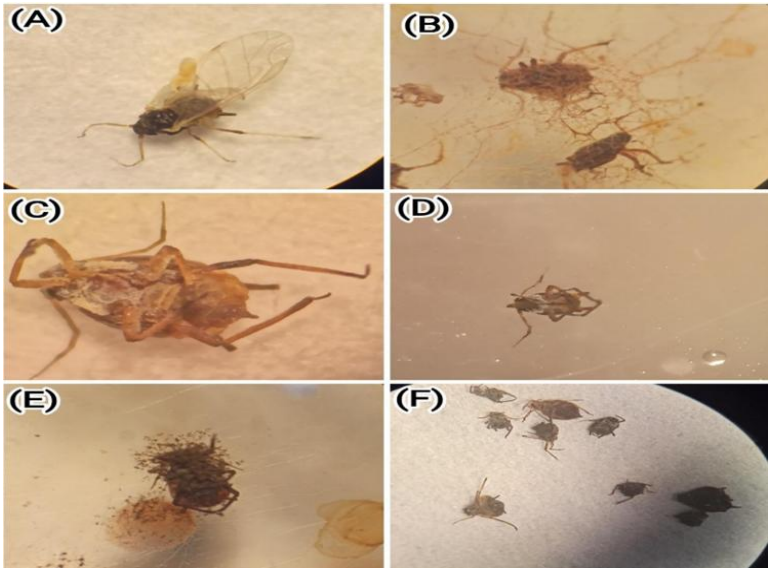

Figure 8. Symptoms of infection in *Aphis spiraecola* caused by different entomopathogenic fungi, observed under a stereomicroscope (X4–5 magnification).A: Winged aphid infected by Cordyceps militaris. B, C, D: Fusarium sp. germinating on aphid (C: adult; D: larva). E: Adult *Aphis spiraecola* infected by *Metarhizium anisopliae*. F: Aphids dipped in *Metarhizium anisopliae.*

These fungi infect their hosts by secreting hydrolytic enzymes that degrade the insect cuticle, facilitating hyphal penetration (Liu et al., 2023; Zhang et al., 2025). The hydrophobic nature of the epicuticle provides an ideal surface for conidial adhesion (Ortiz-Urquiza et al., 2013). Once inside the hemolymph, the fungus must overcome the host's immune defenses to establish infection (Ma et al., 2024).

Toxins such as destruxin E, produced by *Metarhizium anisopliae*, induce rapid yet reversible tetanic paralysis in *Aphis spiraecola*, likely through disruption of calcium ion channels in muscle tissues (Yin et

al., 2023; Millet et al., 2018). Rapid insect death may also result from intestinal ion imbalance, similar to the effects of *Bacillus thuringiensis* toxins (Nébié et al., 2022).

Following host death, the fungus enters a saprophytic phase, producing oosporin to suppress gut-associated bacterial competitors (Cheong et al., 2022; Ma et al., 2024). This stage is marked by insect mummification and sclerotium formation, with the body eventually covered in a white, cottony mycelial mat that initiates conidiospore production (Saiah et al., 2010). The full infection cycle typically spans 3 to 10 days.

3.4 Determination of LT_{50} for Different Treatment

Table 4: Lethal Times of Different Suspensions

LT_{50}	*Metarhizium anisopliae*	*Cordyceps militaris*	*Fusaruim sp*	Positive control (Rustulan)	Negative control (Sterile water)
Larvae	7.84h	14.35h	28.61h	16.28h	85.14h
Adults	25.60h	18.10h	36.33h	21.99h	92.55h

The median lethal time (LT_{50}) values for the three-tested entomopathogenic fungi; *Cordyceps militaris*, *Metarhizium anisopliae*, and *Fusarium sp*. were calculated at a standardized concentration of 1×10^6 conidia/mL against *Aphis spiraecola* populations (Table 4). These values reflect the time required to achieve 50% mortality and serve as a key metric for assessing biocidal speed and efficacy.

All three fungal isolates demonstrated satisfactory LT_{50} values, with notable differences in virulence and speed of action:

For *Cordyceps militaris*: Larvae at14.35 hours; Adults at 18.10 hours

For *Metarhizium anisopliae*: Larvae at 7.84 hours (highest toxicity), Adults at 25.60 hours

For *Fusarium sp*.: Larvae at 61 hours, Adults at 36.33 hours

For comparison, the positive chemical control yielded LT_{50} values of 16.28 hours for larvae and 21.99 hours for adults.

A conidial suspension at 10^6 conidia/ml proved highly effective, yielding significant mortality rates within 72 hours of application (Ghayedi et al., 2013). *Metarhizium anisopliae* and *Cordyceps militaris* exhibited the highest virulence, with LT_{100} values of 24–26 hours for larvae and 36–38 hours for adults. In contrast, *Fusarium sp*. showed lower virulence, with LT_{100} values of 38 hours for larvae and 72 hours for adults.

Statistical analysis revealed no significant differences between the fungal treatments and the commercial insecticide, indicating that the tested biocides are comparably effective under *in vitro* conditions. These results reinforce the potential of *Metarhizium anisopliae* and *Cordyceps militaris* as fast-acting alternatives to chemical control, particularly during the larval stage where susceptibility is highest.

These findings align with those of Saiah et al. (2020), who demonstrated that inoculation technique significantly influences mortality outcomes. For example, *Fusarium sp*. was more effective via dipping, whereas *Metarhizium anisopliae* showed greater virulence when injected. *Metarhizium anisopliae* is widely recognized for its broad-spectrum pathogenicity across multiple insect orders (Lezama-Gutiérrez et al., 2012; Bayissa et al., 2017; Putnoky-Csicsó et al., 2020; Boni et al., 2021; Paradza et al., 2021)

4 CONCLUSIONS

The pathogenicity assays, conducted via foliar application of fungal suspensions on both larval and adult aphid stages, demonstrated the potential of these entomopathogenic fungi as effective biocontrol agents. Among the tested species, *Metarhizium anisopliae* exhibited the highest virulence, followed by *Cordyceps militaris*. In contrast, *Fusarium sp*. showed comparatively lower toxicity and a slower mortality progression.Notably, aphid larvae were significantly more susceptible to fungal infection, with all three entomopathogens achieving a 90% mortality rate within just three days post-treatment. These findings underscore the promise of *M. anisopliae* and *C. militaris* as viable alternatives to conventional chemical insecticides, offering environmentally sustainable options for aphid management.

Despite substantial efforts over recent decades to implement biological control strategies in citrus orchards, the ecological interactions between entomopathogenic fungi and insect hosts remain underexplored. This field likely harbors one of the richest reservoirs of yet-undiscovered fungal species, presenting valuable opportunities for the development of novel biopesticides and integrated pest management solutions.

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