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ARTICLE

Uniting the Role of Entomopathogenic Fungi against *Rhizoctonia solani* JG Kühn, the Causal Agent of Cucumber Damping-Off and Root Rot Diseases

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ABSTRACT

Beauveria bassiana and *Metarhizium* spp. are entomopathogenic fungi with potential applications beyond insect pest control, including plant disease suppression, plant growth promotion, and rhizosphere colonization. This study investigated the plant growth-promoting characteristics and extracellular enzyme activities of *Metarhizium* spp. and *B. bassiana* in relation to phytopathogen interactions and plant growth. Additionally, the efficacy of these fungi in mitigating damping-off and root rot caused by *Rhizoctonia solani* on cucumber plants was evaluated *in vitro* and *in vivo*. Results indicate that *B. bassiana* and *M. anisopliae* produce indole-3-acetic acid, hydrocyanic acid, and hydrolytic enzymes. Seed treatment with these fungi significantly reduced disease severity (3.85%–1.86%, respectively) and enhanced germination parameters [germination percentage (85.33%–86%, respectively), germination index (10.67–12.29, respectively), seedling length vigor index (86.41–109.44, respectively), and seedling weight vigor index (30.24–37.57, respectively)] compared to the control positive. Both fungi demonstrated high inhibition rates of *R. solani* mycelial growth (93.90%–90.46%, respectively). Greenhouse trials revealed that preventive treatments using *B. bassiana* and *M. anisopliae* increased catalase (104.40–105.52 units/mg protein/min, respectively), (4.58–5.77 units/mg protein/min, respectively), superoxide dismutase (40.65–41.74 units/mg protein/min, respectively), and polyphenol oxidase (0.539–0.559 units/mg protein/min, respectively) activities, as well as total phenolic (2.60–2.65 mg/g, respectively) and total sugar content (2.23–2.16 mg/g, respectively) in cucumber plants. Consequently, disease severity (9.51–6.99%, respectively) was reduced, and plant height (93.76–98.76 cm, respectively) increased compared to the positive control. These findings suggest that *B. bassiana* and *M. anisopliae* can enhance plant growth, stimulate plant defense mechanisms, and effectively control damping-off and root rot diseases, making them promising candidates for biological control strategies.

KEYWORDS

Metarhizium spp.; *Beauveria bassiana*; biocontrol; plant growth promotion; *Cucumis sativus*

1 Introduction

Based on scientific projections, the global population is expected to reach between 9.4 and 10.1 billion by 2050 [1]. This significant human population growth, coupled with ongoing climate change and dwindling natural resources, presents a substantial global challenge: ensuring sufficient food supplies for the future [2]. To meet the nutritional demands of this expanding population, total food production will need to increase by an estimated 70% [3]. However, a critical constraint on achieving this goal is the documented scarcity of essential agricultural resources. Multiple scientific publications highlight the diminishing availability of freshwater in many regions [4]. Similarly, agricultural land is becoming increasingly scarce, as reported by various studies [5,6]. Furthermore, supplies of crucial mineral resources for fertilizer production, such as rock phosphate [7] and potassium [8], are also declining. Adding to these challenges is the concern about the rise in the spread of pests and diseases that threaten crop yields [2]. In response to these pressing issues, researchers emphasize the urgent need to adopt sustainable agricultural practices that can minimize plant disease severity [9]. Additionally, experts propose strategies to improve food production through enhanced efficiency in natural resource utilization and a reduction in the environmental impact of fungicides currently used in agriculture [10–12]. Cucumber (*Cucumis sativus* L.) is one of the most widely cultivated and consumed vegetable crops globally. Cucumbers rank among the top four most cultivated vegetables in the world, following only tomatoes, brassicas, and onions [13].

Cucumbers (*Cucumis sativus* L.) are susceptible to a range of fungal diseases, with root rot and damping-off being particularly destructive on a global scale [14]. These diseases are caused by numerous fungal genera, including *Macrophomina phaseolina* (Maublanc) S. Ashby, *Fusarium oxysporum* Schlechtendal, *F. solani* (von Martius) Saccardo, *Phytophthora* spp. De Bary, *Rhizoctonia solani* JG Kühn, and *Sclerotinia rolfsii* Saccardo, as documented in various studies [15–21]. Among these, *R. solani* (teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk) stands out as a major root rot pathogen affecting numerous plant species worldwide [17,19]. While the specific symptoms caused by *R. solani* can vary depending on the host plant, it primarily targets subterranean tissues [22]. Damping-off is the most common symptom associated with *Rhizoctonia* infection, characterized by the inhibition of seed germination and the destruction of seedlings either before or after emergence from the soil [22]. This destructive pathogen is responsible for significant yield losses in both horticultural and crops [17,19,21]. *R. solani* is a destructive soil-borne fungal pathogen responsible for significant crop yield losses globally. Unlike many fungi, *R. solani* lacks asexual spores (conidia) for reproduction. Instead, it relies on sclerotia, hardened survival structures, for propagation and persistence in the soil [19,23]. These sclerotia are considered a primary source of *R. solani* inoculum, facilitating disease spread and infection in susceptible crops [19,23].

The persistent challenge of *R. solani* management stems from its long-lived sclerotia that remain viable in the soil for extended periods, hindering effective control efforts [24]. Despite the implementation of various management strategies, including cultural practices, chemical application, and physical controls, for the management of *R. solani*-induced root rot diseases, overall success has been limited [21]. While crop rotation is a common strategy for managing some plant diseases, the non-specific nature of some *R. solani* strains renders this approach only partially effective [22]. Fungicide use, another common control method, raises concerns about human health risks, environmental pollution, and the potential for pathogen resistance development [9,25]. In recent decades, biological control using various microbes, particularly fungal and bacterial strains, has emerged as a promising alternative for controlling both airborne and soil-borne plant diseases, offering a potential solution for *R. solani* management [26,27].

Beauveria spp. Vuillemin and *Metarhizium* spp. Sorokin, entomopathogenic fungi, have gained significant recognition for their potential in managing various plant pathogens, including *R. solani* [26,28–32]. Beyond their direct antagonistic effect on pathogens, research suggests that incorporating these fungi into crop production systems can promote overall plant health and growth. Studies by [30]

and [33] have shown that *Beauveria* spp. and *Metarhizium* spp. can enhance plant yield and disease resistance. Notably, these fungi have been successfully employed against *R. solani* in a wide range of host plants [27,34]. The mechanisms underlying their antagonistic activity involve both mycoparasitism, where they directly parasitize the pathogen, and the induction of plant resistance [33–36]. Besides, *Beauveria* spp. and *Metarhizium* spp. can colonize the root system without causing harm, while simultaneously stimulating the plant's defense system by increasing the activity of peroxidase and chitinase enzymes, thereby enhancing plant resistance to *R. solani* [27,34]. Additionally, these fungi exhibit faster growth rates compared to fungal plant pathogens. They can further suppress various soil-borne pathogens through the secretion of antifungal compounds. Their application in biological control offers a promising approach to activate plant defense systems and promote growth [37,38]. The present study aimed to investigate the efficacy of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Metarhizium* sp. Me351, in controlling damping-off and root rot diseases in cucumber plants. Additionally, the study sought to evaluate the impact of these entomopathogenic fungi on cucumber seedlings and plant growth, as well as their ability to enhance the plant's defense mechanisms. To achieve these objectives, both *in vitro* and *in vivo* experiments were conducted.

2 Materials and Methods

2.1 Fungal Inoculums Preparation

R. solani was chosen as the target pathogen for both *in vitro* and *in vivo* studies. Three entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) were evaluated as potential biocontrol agents. All cultures originated from the Culture Collection of the Laboratory of Plant Protection (CRRA, Sidi Bouzid, Tunisia). The *R. solani* isolate was obtained from symptomatic cucumber plants, while the entomopathogenic fungi were isolated from cucumber rhizosphere soil collected in agricultural fields of Regueb, Sidi Bouzid. Fungal inocula were prepared from seven-day-old cultures. Briefly, each culture plate was flooded with sterile distilled water. Spores were carefully dislodged using a glass spreader, and the resulting suspension was then filtered with a muslin cloth to eliminate mycelial fragments. The filtered spore suspension was subsequently diluted with sterile distilled water and adjusted to a concentration of 10^7 spores mL^{-1} using a hemocytometer.

2.2 In Vitro Plant-Growth-Promoting and Extracellular Enzymes

This study was conducted under laboratory conditions to assess the potential of three entomopathogenic fungi to promote plant growth. The evaluation focused on essential properties known to benefit plants, including atmospheric nitrogen fixation (N), hydrocyanic acid production (HCN), and indole-3-acetic acid production (IAA). Additionally, the capacity of these fungi to produce extracellular enzymes, namely β-1,3-glucanase (Glu), pectinase (Pec), catalase (Cat), protease (Pro), cellulase (Cell), chitinase (Chi), amylase (Amy), and lipase (Lip) was investigated.

Nitrogen fixation: The atmospheric nitrogen fixation assay employed a Norris Glucose Nitrogen Free (N-free) medium to assess the ability of fungal isolates to utilize atmospheric nitrogen as a nitrogen source. Isolates capable of N fixation were expected to grow and use atmospheric nitrogen for their metabolic needs. The entomopathogenic fungi were incubated at 30°C for 5 days. This growth manifested as a visible film on the surface of the N-free medium [39].

Hydrocyanic acid production: HCN production by the entomopathogenic fungi was evaluated using a qualitative assay adapted from [9]. This method assesses the cyanogenic potential of the fungi, which refers to their ability to produce HCN. Agar plates containing 15 mL of PDA supplemented with 4.4 g/L glycine were inoculated with a one-disc plug. The lids of these dishes are modified to include a filter paper (diameter 9 cm) impregnated with an alkaline picrate solution placed underneath. The plates were incubated at 28°C ± 2°C for 4 days. HCN production by the fungi, if present, would cause a color

change in the filter paper from its original yellow to a reddish-brown hue. This color change is a positive indicator of HCN production by the fungi [9].

Indole-3-acetic acid production: IAA production by the entomopathogenic fungi was evaluated using a qualitative colorimetric assay adapted from [9]. This assay assesses the ability of the fungi to synthesize IAA, a plant growth hormone. The method involves placing a one-disc plug of fungi on the Luria-Bertani medium. Following incubation (28°C for 48 h), a Whatman paper disk (diameter 5 cm) pre-treated with Salkowski's reagent is placed on the culture surface. Salkowski's reagent reacts with IAA to produce a color change. If the fungi produce IAA, the filter paper will change from yellow to a pinkish-brown hue. This color shift is a positive indicator of IAA production by the fungi [9].

β-1,3-glucanase activity: The ability of the entomopathogenic fungi to produce Glu was evaluated using a clear zone formation assay based on the method described by [40]. This assay assesses the presence and activity of Glu enzymes, which can degrade β-1,3-glucan polysaccharides. The assay utilizes solidified agar plates containing laminarin (1 g/L), a β-1,3-glucan substrate, along with peptone (0.5 g/L) and yeast extract (0.1 g/L) for fungal growth. A fungal plug was inoculated onto the agar surface and incubated at 28°C. If the fungi produce Glu, the enzyme will diffuse from the colony and hydrolyze the surrounding laminarin. This degradation creates a clear halo zone around the fungal colony. The absence of a stainable substrate in this zone allows for easy visualization upon observation. The size of the clear zone can be correlated with the level of Glu activity produced by the fungi [40].

Pectinase production: The pectinase activity of the entomopathogenic fungi was assessed using a zone-formation assay based on the method described by [41]. Pectinase enzymes degrade pectin, a major component of plant cell walls. The assay employs solidified agar plates containing pectin as the substrate for potential pectinase activity. A fungal plug was inoculated onto these plates and incubated at 28°C for five days. Following incubation, the plates are flooded with a 0.05% ruthenium red solution. Ruthenium red acts as a specific stain for pectin, binding to it and causing the medium to turn red. After staining, the plates are thoroughly rinsed with distilled water to remove unbound ruthenium red. A clear halo zone surrounding the fungal colonies after washing signifies pectinase production by the isolates. This clear zone represents the area where pectin has been degraded by the secreted pectinases. The formation of this halo zone demonstrates the enzymatic activity of the fungi and their potential role in plant tissue maceration, facilitating nutrient acquisition from the degraded plant material [41].

Catalase production: Catalase activity of the entomopathogenic fungi was assessed using a qualitative slide test adapted from [39]. Catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water and oxygen. This assay employs a simple and rapid method to determine the presence of catalase activity in the fungal isolates. A small drop of H_2O_2 solution is mixed with a sample of the fungal colony and placed on a microscope slide. If the fungi possess catalase activity, the H_2O_2 will be rapidly broken down, resulting in the production of visible gas bubbles. The formation of these air bubbles serves as a positive indicator of catalase activity. This assay provides a quick and easy method for preliminary screening of catalase activity in fungal isolates [39].

Protease production: The protease activity of the entomopathogenic fungi was assessed using a zone-formation assay on skim milk agar medium. Skim milk agar is a commonly employed medium for detecting protease activity as it contains casein, a milk protein that serves as a substrate for proteases. A fungal plug was inoculated onto the solidified plates and incubated at 28°C for five days. Following incubation, the plates were observed for a clear halo zone surrounding the fungal colonies. The formation of this clear zone is indicative of protease production by the entomopathogenic fungi. This zone represents the area where casein has been hydrolyzed (broken down) by the secreted proteases. The observed enzymatic activity demonstrates the ability of the fungi to degrade proteins, which can be beneficial in various applications [42].

Cellulase production: The cellulase activity of the entomopathogenic fungi was evaluated using a zone-formation assay on carboxymethylcellulose (CMC) agar medium. CMC serves as a surrogate substrate for cellulose, a major component of plant cell walls that can be degraded by cellulase enzymes. The assay employs solidified CMC agar plates inoculated with one-disc plug of fungi and incubated at 28°C for five days. Following incubation, the plates are flooded with a 1% Congo red solution. Congo red is a dye with a high affinity for cellulose, staining it red. However, cellulase activity disrupts this binding. After staining, the plates are thoroughly washed with distilled water to remove unbound Congo red. The formation of a clear halo zone surrounding the fungal colonies after washing signifies cellulase production by the isolates. This clear zone represents the area where cellulose has been degraded by the secreted cellulases, preventing Congo red from binding. This reduction in stain binding demonstrates the enzymatic activity of the fungi and their potential role in cellulose breakdown [43].

Chitinase production: The chitinase activity of the entomopathogenic fungi was assessed using a zone-formation assay based on the method described by [44]. Chitinase enzymes break down chitin, a polysaccharide that is a major structural component of phytopathogenic fungi and bacteria cell walls and the exoskeletons of insects. The assay utilizes a solidified agar medium containing colloidal chitin as the substrate for potential chitinase activity. A fungal plug was inoculated onto these plates and incubated at 28°C for 5 days. Following incubation, the plates are observed for a clear halo zone surrounding the fungal colonies. The formation of this clear zone signifies chitinase production by the fungal isolates. This zone represents the area where chitin has been degraded by the secreted chitinases. The observed enzymatic activity demonstrates the potential of the fungi to weaken the cell walls of phytopathogenic fungi and bacteria or decompose insect remains through chitin breakdown [44].

Amylase production: The amylase activity of the entomopathogenic fungi was evaluated using a zone-formation assay on a soluble starch-containing agar medium. Starch is a complex carbohydrate found in many organisms and can be broken down into simpler sugars by amylase enzymes. The assay employs solidified agar plates containing soluble starch as the substrate for potential amylase activity. A fungal plug was inoculated onto these plates and incubated at 28°C for 5 days. Following incubation, the plates are flooded with an iodine solution. Iodine has a high affinity for starch, readily binding and forming a characteristic blue-black complex. However, amylase activity disrupts this interaction. If the fungi produce amylase, the enzyme will hydrolyze the surrounding starch into simpler sugars that cannot bind iodine. After staining with iodine, the plates are observed for a clear halo zone surrounding the fungal colonies. The formation of this clear zone signifies positive amylase production by the entomopathogenic fungi. This zone represents the area where starch has been degraded by the secreted amylases, preventing iodine binding. The observed enzymatic activity demonstrates the ability of the fungi to break down starch, which can play a role in their carbohydrate metabolism and nutrient acquisition [45].

Lipase production: The lipase activity of the entomopathogenic fungi was evaluated using a zone-formation assay on a solid medium containing Tween 80. Tween 80 is a synthetic substrate commonly used in lipase assays as it mimics the structure of natural fats and oils. A fungal plug was inoculated onto the solidified plates and incubated at 28°C. Lipase enzymes, if produced by the fungi, can hydrolyze (break down) Tween 80 into simpler components. Following incubation, the plates were observed for the formation of an opaque halo zone surrounding the fungal colonies. The presence of this opaque zone signifies positive lipase production by the isolates. This zone represents the area where Tween 80 has been hydrolyzed by the secreted lipases. The hydrolysis process produces fatty acids, which are insoluble in the agar medium. These insoluble fatty acids precipitate and scatter light, resulting in the opaque appearance of the halo zone [9].

2.3 Cucumber Seed Germination Assay

To assess the effects of three entomopathogenic fungi on cucumber seed germination and their potential interaction with the fungal pathogen *R. solani*, a germination assay was conducted. Healthy cucumber seeds were surface-sterilized using a 2% sodium hypochlorite solution for 3 min, rinsed thoroughly with distilled water, and dried under laminar airflow on sterilized blotting paper. Subsequently, the sterilized seeds were treated by submersion in flasks containing conidial suspensions of each entomopathogenic fungus for 30 min. After treatment, all seeds were placed in a moist chamber at 98% relative humidity and 25°C–28°C for 24 h, followed by air-drying. After 24 h incubation, seeds were individually inoculated with *R. solani*. Each seed received a single 10 µL droplet of the *R. solani* inoculum. Two positive controls were established: one inoculated with only the pathogen and another treated solely with the entomopathogenic fungi suspension (10 mL). Distilled water served as the negative control. The experiment employed eight treatments: T1—untreated seeds (negative control), T2—seeds treated only with *R. solani* (positive control), T3—seeds treated only with *B. bassiana*, T4—seeds treated only with *M. anisopliae*, T5—seeds treated with *Metarhizium* sp. Me351, T6—seeds treated with *B. bassiana* and inoculated with *R. solani*, T7—seeds treated with *M. anisopliae* and inoculated with *R. solani*, and T8—seeds treated with *Metarhizium* sp. Me351 and inoculated with *R. solani*. Each treatment was replicated four times, resulting in 160 Petri dishes [8 treatments × 4 replicates (each replicate containing 5 Petri dishes)]. Each Petri dish (9 cm) contained 10 cucumber seeds placed on a layer of absorbent cotton wool and sterilized blotting paper. For germination, the dishes were incubated at 25°C under a controlled light regime of 16 h light and 8 h dark.

Seed germination was assessed based on radicle emergence and a minimum length exceeding 2.0 mm. Fifteen days post-inoculation (dpi), seedlings were collected from each treatment group to evaluate various parameters. These parameters included disease progression [disease severity index (DSI) and percent of infected seeds (PIS)] as outlined by [46] and [47], and germination metrics [germination percentage (GP), germination index (GI), seedling length vigor index (SLVI), and seedling weight vigor index (SWVI)] as described by [48]. Disease index was assessed using a 0–4 scale adapted from [46], where 0 indicates no visible damage, 1 signifies minor discoloration of the hypocotyl, 2 represents discoloration combined with small necrotic lesions (less than 1 mm diameter) on the hypocotyl, 3 indicates discoloration with larger necrotic lesions (greater than or equal to 1 mm diameter) on the hypocotyl, and 4 signifies seedling death. Disease severity data were then processed using McKinney's formula [49] to generate a numerical DSI: DSI (%) = (Σvn)/(NV) × 100. In this formula, Σvn represents the sum of the product obtained by multiplying the disease index score (v) by the number of plants (n) assigned to that score. N represents the total number of plants in the experiment, and V represents the highest numerical value on the disease index scale. The efficacy of each entomopathogenic fungi treatment was subsequently rated based on the calculated DSI using the classification system established by [25]. This classification system categorizes treatment efficacy as follows: EE: Extremely effective (DSI = 0%), HE: Highly effective (DSI = 0.1% to 5%), E: Effective (DSI = 5.1% to 25%), I: Ineffective (DSI = 25.1% to 50%), and HI: Highly Ineffective (DSI = 50.1% to 100%). PIS = Total number of infected seedlings/Total seeds sown × 100 [47]. Germination% = Total number of germinated seeds/Total seeds sown × 100 [48]. GI = Total number of germinated seeds/Total number of days [48]. SLVI = [seedling length (cm) × seed germination (%)] [48]. SWVI = [seedling DW (mg) × seed germination (%)] [48].

2.4 Antagonistic Action of Entomopathogenic Fungi toward Rhizoctonia solani

The antagonistic interaction between the entomopathogenic fungi and the *R. solani* pathogen was evaluated using a dual culture assay on potato dextrose agar (PDA) plates. Two agar plugs (0.5 cm) were obtained: one containing a four-day-old culture of the entomopathogenic fungus and another containing the *R. solani* pathogen. These plugs were placed on opposing sides of a single 9-cm diameter PDA plate,

maintaining a distance of 2 cm from the plate edge towards the center for the antagonist plug and a distance of 5 cm between the two plugs. A control plate included only a PDA plug on one side and the *R. solani* plug on the opposite side. Each treatment was replicated three times, with five plates per replicate. All plates were incubated at 28°C ± 2°C for 7 days. After incubation, the percent inhibition of *R. solani* radial growth was calculated using the formula established by [50]: I (%) = (1 − Cn/C0) × 100. In this formula, Cn represents the radial growth of the pathogen colony in the presence of the antagonist fungus, and C0 represents the radial growth of the control pathogen colony. Mycelial growth was assessed in cm as described in [49].

2.5 Greenhouse Evaluation of Entomopathogenic Fungi against Rhizoctonia solani

The *in vivo* experiment investigated the potential of entomopathogenic fungi for the preventive control of *R. solani* in cucumber plants. Healthy cucumber seeds were sown in nursery trays containing cells with a 250 mL volume. Each treatment consisted of 15 plants, further divided into three replicates with five plants each. The substrate for the experiment was a sterilized 1:1 mixture of peat and vermiculite, achieved by autoclaving twice at 120°C. Thirty-day-old cucumber seedlings were then root-dipped in flasks containing conidial suspensions of the different entomopathogenic fungi for 30 min as a preventive treatment. Twenty-four hours after this treatment, the seedlings were challenged with *R. solani* by watering each plant with 10 mL of the pathogen's conidial suspension. Each experimental block included two control groups: a positive control, where plants were inoculated only with the *R. solani* pathogen, and a negative control, where plants were treated solely with sterile distilled water. The experiment employed a randomized complete block design with eight treatments: T1 (negative control)—untreated seedlings; T2 (positive control)—seedlings inoculated only with *R. solani*; T3—seedlings treated only with *B. bassiana*, T4—seedlings treated only with *M. anisopliae*, T5—seedlings treated only with *Metarhizium* sp. Me351, T6—seedlings treated with *B. bassiana* and inoculated with *R. solani*, T7—seedlings treated with *M. anisopliae* and inoculated with *R. solani*, and T8—seedlings treated with *Metarhizium* sp. Me351 and inoculated with *R. solani*. After treatment, the pots were incubated in a greenhouse for 60 days (at 25°C). Post-experimental, the tomato plants were carefully removed from their pots. The root systems were subsequently rinsed thoroughly with tap water. The entire experiment was repeated twice to ensure data robustness [49,51].

Disease assessment was conducted at 60 dpi using a DSI using McKinney's formula [49]. The efficacy of each entomopathogenic fungi treatment was subsequently rated based on the calculated DSI using the classification system established by [50] and further refined by [52]. Plant growth was assessed by measuring plant length (PL) [25].

To elucidate the biochemical alterations induced in cucumber plants by the preventive application of *B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351, a series of biochemical parameters were evaluated. Enzyme activity assays were conducted on five cucumber root samples collected per treatment and block, at 60 dpi. These assays measured the activity of catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), and polyphenol oxidase (PPO). Additionally, total phenolic content (TPC) and total sugar (TS) levels were quantified in the root tissues.

To prepare enzyme extracts for activity assays, 0.1 g root samples from each treatment were flash-frozen in liquid nitrogen to inhibit proteolytic activity. The frozen samples were then homogenized in a chilled extraction buffer (0.1 M phosphate buffer with 0.5 mM EDTA, pH 7.5) at 1:5 (buffer:tissue weight). The homogenate was centrifuged at 15,000× *g* for 20 min at 4°C. The resulting supernatant was collected in Eppendorf tubes for subsequent enzyme activity assays. CAT activity was measured according to the method described by [53], monitoring the decrease in absorbance at 240 nm. POX activity was assayed following the protocol established by [54]. The reaction mixture for POX activity consisted of 0.5 mL guaiacol, 1 mL phosphate buffer, 0.5 mL H_2O_2, 0.1 mL enzyme extract, and 0.9 mL distilled water.

The change in absorbance was measured at 470 nm. SOD activity was determined using the method of [55]. The reaction mixture for SOD activity contained 130 μM L-methionine, 13 μM riboflavin, 50 mM phosphate buffer (pH 7.0), 630 μM NBT (nitro blue tetrazolium), EDTA disodium salt (EDTAna2), and enzyme extract. The absorbance of this mixture was monitored at 560 nm. PPO activity was evaluated according to the method of [56], measuring the increase in absorbance at 408 nm. The reaction mixture for PPO activity comprised 2.7 mL of 14 mg mL^{-1} catechol solution in 0.1 M phosphate buffer and 0.3 mL of enzyme extract [56].

TPC and TS levels were determined in cucumber root tissues. TPC was measured using the Folin-Ciocalteu method, where the absorbance of the reaction mixture was measured at 765 nm. TPC was then expressed as milligrams of gallic acid equivalent per gram of fresh weight (mg GAE/g FW) according to the method established by [57]. For TS quantification, the anthrone method was employed, with absorbance readings taken at 625 nm. The TS content was expressed based on the extrapolation from a linear standard curve prepared using glucose [58].

2.6 Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) implemented in SPSS version 20.0 software to evaluate potential variations among the different treatment groups. Before conducting the ANOVA, data from replicate samples were averaged to obtain representative mean values for each treatment. The assumptions of normality and homogeneity of variance were verified using appropriate tests before proceeding with the ANOVA. Subsequently, Duncan's Multiple Range Test was employed to identify statistically significant differences ($p \leq 0.05$) between the treatment means. This *post-hoc* test allowed for a nuanced comparison of the treatment effects and identifying important variations in the measured parameters across the different entomopathogenic fungi treatments and control groups.

3 Results

3.1 In Vitro Plant-Growth-Promoting Traits and Extracellular Enzymes Activities

An assessment of extracellular enzyme production and plant growth promotion capabilities was conducted for three entomopathogenic fungi. Table 1 presents the detailed results of enzyme activity and plant growth effects. Both *B. bassiana* and *M. anisopliae* exhibited a wide range of extracellular enzyme activities. These two fungi showed positive activities for Glu, Pec, Cat, Pro, Cell, Chi, Amy, and Lip. However, *Metarhizium* sp. Me351 showed a more selective enzyme profile. It demonstrated positive activities for Glu, Pec, Cat, and Cell but lacked Pro, Chi, Amy, and Lip (Table 1). *B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351 showed positive traits for HCN and IAA production; crucial for plant growth promotion. However, none of the isolates exhibited atmospheric nitrogen fixation ability, as indicated by the negative results in the N-free medium (Table 1).

Table 1: Extracellular enzyme activities and plant-growth-promoting traits of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) after 5 days of incubation at 28°C

Isolates	*B. bassiana*	*M. anisopliae*	*Metarhizium* sp. Me351
N	−	−	−
HCN	+	+	+
IAA	+	+	+
Glu	+	+	+
Pec	+	+	+

(Continued)

Table 1 (continued)

Isolates	*B. bassiana*	*M. anisopliae*	*Metarhizium* sp. Me351
Cat	+	+	+
Pro	+	+	−
Cell	+	+	+
Chi	+	+	−
Amy	+	+	−
Lip	+	+	−

Note: N: atmospheric nitrogen fixation (N-free medium); HCN: hydrocyanic acid production (Potato dextrose agar medium); IAA: indole-3-acetic acid production (Luria-Bertani agar culture medium); Glu: β-1,3-glucanase activity (enriched medium with β-1,3-glucanase); Pec: pectinase production (Pectino-Congo Red agar medium); Cat: catalase production; Pro: protease production (skim milk agar medium); Cell: cellulase production (carboxymethylcellulose agar medium); Chi: chitinase production (agar medium containing colloidal chitin); Amy: amylase production (agar medium containing soluble starch); Lip: lipase production (agar medium containing Tween 80).

3.2 Cucumber Seed Germination Assay

The effectiveness of entomopathogenic fungi on the progression of disease caused by *R. solani* in cucumber seeds is summarized in Table 2. Seeds without any treatment (negative control) and those treated with just entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) showed no signs of disease (DSI = 0%). This indicates they are extremely effective (EE) at preventing the disease. In contrast, seeds inoculated only with the disease-causing fungus (positive control) displayed severe symptoms (DSI = 97.33%), making them highly ineffective (HI). When these beneficial fungi (*B. bassiana* and *M. anisopliae*) were combined with the harmful fungus, they significantly reduced disease severity (DSI = 3.85% and 1.86%, respectively), making them highly effective (HE). *Metarhizium* sp. Me351, while effective (E) at reducing disease (DSI = 27.77%), provided less protection compared to the other two beneficial fungi (Table 2).

Table 2: Effect of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) against *R. solani* on the disease progression [disease severity index (DSI), protective potential of treatments (ET), and percent of infected seeds (PIS)] after 15 days of post-inoculation under controlled conditions in a growth chamber (8 h dark/16 h light photoperiod at 20°C–22°C)

Treatments	DSI (%)	ET	PIS (%)
Negative control	0 ± 0c[a]	EE	0 ± 0d
Positive control	97.33 ± 0.18a	HI	96.33 ± 0.52a
B. bassiana	0 ± 0c	EE	0 ± 0d
M. anisopliae	0 ± 0c	EE	0 ± 0d
Metarhizium sp.	0 ± 0c	EE	0 ± 0d
B. bassiana + *R. solani*	3.85 ± 0.37c	HE	3.67 ± 0.09c
M. anisopliae + *R. solani*	1.86 ± 0.05c	HE	1.67 ± 0.13cd
Metarhizium sp. + *R. solani*	27.77 ± 0.72b	E	22 ± 0.56b
***p-value*[b]**	**<0.01**	**Nd**	**<0.01**

Note: [a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at $p \leq 0.05$. [b]Probabilities associated with individual F tests. EE: Extremely effective; HE: Highly effective; E: Effective; I: In-effective; HI: Highly in-effective; Nd: Not determined. Average values ± standard deviation; Nd: Not determined. EE: Extremely effective (DSI = 0%), HE: Highly effective (DSI = 0.1% to 5%), E: Effective (DSI = 5.1% to 25%), I: Ineffective (DSI = 25.1% to 50%), and HI: Highly ineffective (DSI = 50.1% to 100%).

The negative control group, representing seeds without treatment, had a 0% infection rate, as expected. Interestingly, treatments containing only entomopathogenic fungi also resulted in a 0% infection rate. This suggested that these fungal strains themselves are not harmful to the seeds and might even possess some protective properties. In contrast, the positive control group, representing seeds inoculated with *R. solani*, exhibited a high infection rate of 96.33%. When either *B. bassiana* or *M. anisopliae* was combined with *R. solani*, the seed infection rate dropped significantly. The combination with *B. bassiana* resulted in a 3.67% infection rate, while the combination with *M. anisopliae* achieved an even lower infection rate of 1.67%. These significant reductions in infection rate compared to the positive control group demonstrated the effectiveness of this combined approach. However, *Metarhizium* sp. Me351, when combined with *R. solani*, resulted in a higher infection rate (22%) than other combinations. While this infection rate is still significantly lower than the positive control, it suggests that *Metarhizium* sp. Me351 might be less effective in this specific context (Table 2).

Table 3 delved into the effects of entomopathogenic fungi on seed germination and seedling development after exposure to *R. solani*. The experiment was conducted under controlled conditions in a growth chamber for 15 days after inoculation. The negative control group, representing seeds without treatment, displayed a high GP (85.67%) and GI (8.57), indicating successful seed development. The seedling length vigor index (SLVI = 106.85) and seedling weight vigor index (SWVI = 32.12) further confirm healthy seedling growth in the negative control. When compared to the negative control group, seeds in the positive control group exhibited significantly lower values for all measured parameters: GP (42.33%), GI (2.19), SLVI (74.70), and SWVI (27.99). These findings suggested that *R. solani* infection disrupted physiological processes for successful seed germination and seedling development. When applied alone, *B. bassiana* or *M. anisopliae* did not negatively affect germination, with germination metrics similar to the negative control. Thus, both entomopathogenic fungi maintained high GP exceeding 88% and GI above 11.04. Moreover, *B. bassiana* and *M. anisopliae* exhibited a significantly higher value of SLVI (111.69 and 109.07, respectively), and SWVI (34.25 and 31.62, respectively), suggesting robust seedling development. However, *Metarhizium* sp. Me351 displayed a slight decrease in all germination metrics (GP = 80.33%, GI = 8.93, SLVI = 100.02, SWVI = 32.19) compared to the other entomopathogenic fungi. Moving on to the combination treatments, both *B. bassiana* and *M. anisopliae* partially mitigated the negative effects of *R. solani* on germination. They achieved GP (85.33% and 86%, respectively), GI (10.67 and 12.29, respectively), SLVI (86.41 and 109.44, respectively), and SWVI (30.24 and 37.57, respectively) closer to the negative control compared to the positive control. However, *Metarhizium* sp. Me351, in the presence of *R. solani*, offered less protection for germination metrics (Table 3).

Table 3: Effect of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) against *R. solani* on the germination metrics [germination percentage (GP), germination index (GI), seedling length vigor index (SLVI), and seedling weight vigor index (SWVI)] after 15 days of post-inoculation under controlled conditions in a growth chamber (8 h dark/16 h light photoperiod at 20°C–22°C)

Treatments	GP (%)	GI	SLVI	SWVI
Negative control	85.67 ± 0.87ab[a]	8.57 ± 0.07d	106.85 ± 0.68a	32.12 ± 0.33abc
Positive control	42.33 ± 0.75c	2.19 ± 0.56e	74.70 ± 0.81b	27.99 ± 0.37c
B. bassiana	88.33 ± 0.32a	12.62 ± 0.42a	111.69 ± 0.96a	34.25 ± 0.75ab
M. anisopliae	88.33 ± 0.55a	11.04 ± 0.32bc	109.07 ± 1.02a	31.62 ± 1.01abc
Metarhizium sp.	80.33 ± 0.69ab	8.93 ± 0.87d	100.02 ± 1.08ab	32.19 ± 0.28abc

(Continued)

Table 3 (continued)

Treatments	GP (%)	GI	SLVI	SWVI
B. bassiana + *R. solani*	85.33 ± 0.19ab	10.67 ± 0.48c	86.41 ± 0.44ab	30.24 ± 0.94bc
M. anisopliae + *R. solani*	86 ± 0.28ab	12.29 ± 0.65ab	109.44 ± 0.72a	37.57 ± 0.59a
Metarhizium sp. + *R. solani*	75 ± 0.47b	9.38 ± 0.17d	78.87 ± 0.53b	29.44 ± 0.26bc
***p-value*[b]**	**<0.01**	**<0.01**	**<0.05**	**<0.05**

Note: [a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at $p \leq 0.05$. [b]Probabilities associated with individual F tests. Average values ± standard deviation.

3.3 Antagonistic Action of Entomopathogenic Fungi toward Rhizoctonia solani

The experiment investigated the impact of entomopathogenic fungi on the mycelial growth of *R. solani*. Significant effects ($p < 0.01$) were observed on both *R. solani* growth and the percentage of inhibition of its radial growth (Figs. 1 and 2). Among the entomopathogenic fungi tested, *B. bassiana* and *M. anisopliae* displayed the strongest inhibition of *R. solani* growth, with radial growth values of 0.31 cm and 0.49 cm, respectively. Conversely, the control group exhibited the highest radial growth at 5.13 cm (Fig. 1). Similarly, *B. bassiana* and *M. anisopliae* resulted in the greatest percentage inhibition of *R. solani* mycelial growth, achieving values between 93.90% and 90.46% (Fig. 2).

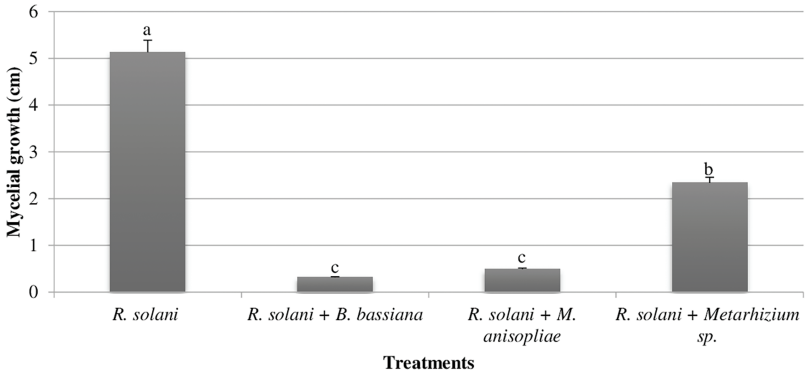

Figure 1: Effect of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) on mycelial growth of *R. solani* after 7 days of incubation at 28 ± 2°C under laboratory conditions. Potato Dextrose Agar medium solidified in 9 cm diameter Petri dishes served as the growth substrate for the fungal isolates. Different letters above bars indicate statistically significant differences between treatments within the experiments ($p \leq 0.01$) according to Duncan's multiple range tests

3.4 Greenhouse Evaluation of Entomopathogenic Fungi against Rhizoctonia solani

The efficacy of entomopathogenic fungi in controlling *R. solani* was evaluated *in vivo* by assessing the DSI in cucumber roots. The results revealed a significant suppressive effect of *B. bassiana* against *R. solani*, with a DSI of 9.51% compared to the positive control group experiencing a severe disease level (DSI = 99.33%). Furthermore, *M. anisopliae* demonstrated even greater efficacy, achieving a remarkably low DSI of 6.99%. *Metarhizium* sp. Me351, although exhibiting a less effect (DSI = 40.19%) compared to the other two fungi, still displayed a statistically significant reduction in disease severity compared to the untreated control group. These findings suggest that all three entomopathogenic fungi possess potential for the biological control of *R. solani* in cucumber, with *M. anisopliae* demonstrating the most promising results in this study (Fig. 3).

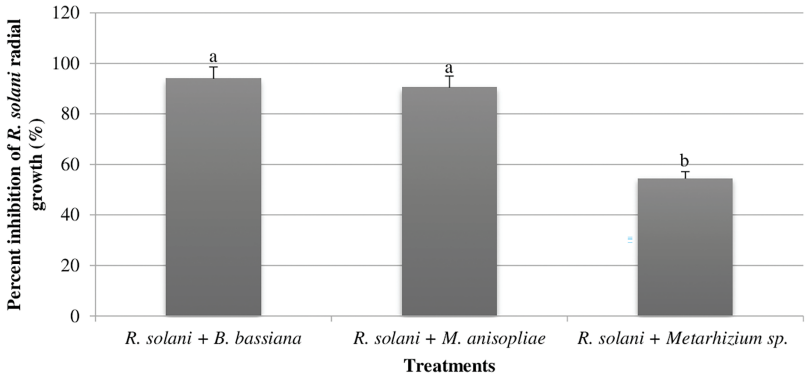

Figure 2: Effect of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) on the percent inhibition of *R. solani* radial growth after 7 days of incubation at 28 ± 2°C under laboratory conditions. Potato Dextrose Agar medium solidified in 9 cm diameter Petri dishes served as the growth substrate for the fungal isolates. Different letters above bars indicate statistically significant differences between treatments within the experiments ($p \leq 0.01$) according to Duncan's multiple range tests

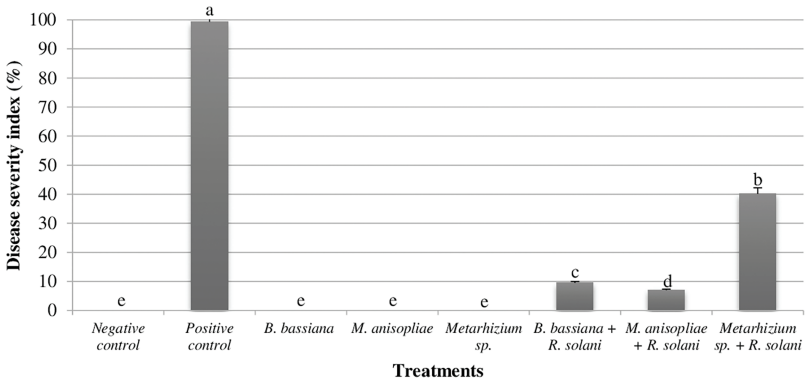

Figure 3: *In vivo* evaluation of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) on the disease severity index in cucumber roots inoculated with *R. solani* after 60 days of growth under greenhouse conditions at 25°C. Different letters above bars indicate statistically significant differences between treatments within the experiments ($p \leq 0.01$) according to Duncan's multiple range tests

This study also evaluated the effects of entomopathogenic fungi on cucumber plant growth against *R. solani*. After 60 days of controlled greenhouse growth, plant length was measured. Uninoculated and untreated plants (negative control) exhibited moderate growth (83.15 cm). However, plants inoculated with *R. solani* alone (positive control) displayed the most severe growth reduction (35.33 cm). Thus, treatment with *B. bassiana* resulted in the greatest plant height (140.79 cm), indicating significant growth promotion. *Metarhizium* treatments (Me351: 140.77 cm and *M. anisopliae*: 139.94 cm) also significantly enhanced plant growth with lengths comparable to *B. bassiana*. When co-inoculated with *R. solani*, *M. anisopliae* (98.76 cm) and *B. bassiana* (93.76 cm) treatments displayed reduced plant length compared to entomopathogenic fungi-only treatments but remained significantly taller than controls. *Metarhizium* sp. Me351 co-inoculation led to the lowest plant height within the fungi-treated groups (59.66 cm) but surpassed the positive control (Fig. 4).

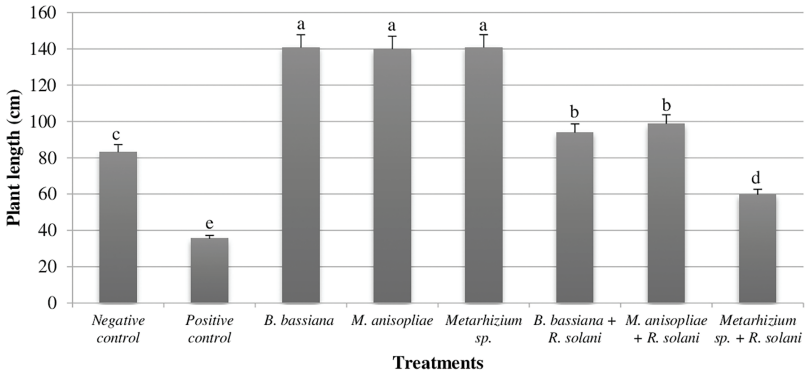

Figure 4: *In vivo* evaluation of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) on the plant length of cucumber plants inoculated with *R. solani* after 60 days of growth under greenhouse conditions at 25°C. Different letters above bars indicate statistically significant differences between treatments within the experiments ($p \leq 0.01$) according to Duncan's multiple range tests

Table 4 revealed the effect of three entomopathogenic fungi in the presence of *R. solani* on CAT, POX, SOD, PPO, TPC, and TS. *B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351 treatments displayed an average CAT activity of 70.03, 70.77, and 69.41 units/mg protein/min, respectively, compared to the negative (14.94 units/mg protein/min) and positive (23.60 units/mg protein/min) controls. Similarly, POX activity more than tripled in the presence of entomopathogenic fungi, with an average of 3.24, 3.45, and 3.33 units/g/mL/min for *B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351, respectively, compared to the negative (1.13 units/mg protein/min) and positive (1.52 units/mg protein/min) controls. Furthermore, the result revealed an interaction between the entomopathogenic fungi and *R. solani*. When these fungi were co-inoculated with *R. solani*, CAT activity witnessed a further significant increase compared to the treatments with entomopathogenic fungi alone. Interestingly, *M. anisopliae* + *R. solani* exhibited the highest CAT activity (105.52 units/mg protein/min) among all treatments. A similar trend was observed for POX activity, with the *M. anisopliae* + *R. solani* treatment showing the highest value (5.77 units/mg protein/min) (Table 4). The combination of entomopathogenic fungi and *R. solani* (*B. bassiana* + *R. solani*, *M. anisopliae* + *R. solani*) resulted in the highest SOD (40.65 and 41.74 units/mg protein/min, respectively) and PPO (0.539 and 0.559 units/mg protein/min, respectively) activity, while *Metarhizium* sp. + *R. solani* showed an intermediate effect (34.96 units/mg protein/min and 0.383 units/mg protein/min, respectively) (Table 4). All fungal treatments significantly ($p < 0.01$) increased TPC compared to the negative (1.33 mg/g) and positive (1.64 mg/g) controls. The highest TPC levels were observed in plants treated with *M. anisopliae* (2.65 mg/g) and *B. bassiana* (2.60 mg/g) in the presence of *R. solani* (Table 4). The fungal treatments generally resulted in increased TS compared to the controls. Moreover, the combination of *B. bassiana* (2.23) and *M. anisopliae* (2.16) with *R. solani* showed an increase in TS (Table 4).

Table 4: Effect of entomopathogenic fungi (*B. bassiana*, *M. anisopliae*, and *Metarhizium* sp. Me351) against *R. solani* on the activities of catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), and polyphenol oxidase (PPO), and the contents of total phenolic (TPC) and total sugar (TS) after 60 days of growth under greenhouse conditions at 25°C

Treatments	CAT (units/mg protein/min)	POX (units/g/mL/min)	SOD (units/g/mL/min)	PPO (units/g/mL/min)	TPC (mg/g)	TS (mg/g)
Negative control	14.94 ± 0.11e [a]	1.13 ± 0.01d	16.01 ± 0.75d	0.129 ± 0.01f	1.33 ± 0.09d	1.30 ± 0.08d

(Continued)

Table 4 (continued)

Treatments	CAT (units/mg protein/min)	POX (units/g/mL/min)	SOD (units/g/mL/min)	PPO (units/g/mL/min)	TPC (mg/g)	TS (mg/g)
Positive control	23.60 ± 0.43d	1.52 ± 0.14d	24.41 ± 1.01c	0.244 ± 0.01e	1.64 ± 0.17c	1.51 ± 0.01c
B. bassiana	70.03 ± 0.18c	3.24 ± 0.09c	34.90 ± 1.16b	0.334 ± 0.02cd	1.94 ± 0.08b	1.91 ± 0.08b
M. anisopliae	70.77 ± 0.12c	3.45 ± 0.02c	35.02 ± 0.87b	0.365 ± 0.01bc	1.97 ± 0.06b	1.91 ± 0.11b
Metarhizium sp.	69.41 ± 0.94c	3.33 ± 0.01c	30.58 ± 0.63	0.313 ± 0.03d	1.88 ± 0.14b	1.86 ± 0.19b
B. bassiana + *R. solani*	104.40 ± 1.51a	4.58 ± 0.03b	40.65 ± 0.54a	0.539 ± 0.03a	2.60 ± 0.12a	2.23 ± 0.22a
M. anisopliae + *R. solani*	105.52 ± 2.01a	5.77 ± 0.02a	41.74 ± 0.96a	0.559 ± 0.01a	2.65 ± 0.21a	2.16 ± 0.17a
Metarhizium sp. + *R. solani*	81.70 ± 1.17b	3.64 ± 0.11c	34.96 ± 0.81b	0.383 ± 0.02b	1.87 ± 0.03b	1.61 ± 0.01c
p-value [b]	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**	**< 0.01**

Note: [a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at $p \leq 0.01$. [b]Probabilities associated with individual F tests. Average values ± standard deviation.

Table 5 detailed the correlation coefficients between various indicators of plant growth, stress response, and disease severity measured after 60 days of controlled greenhouse growth. The table highlighted significant positive correlations ($r > 0.8$) between the activities of four antioxidant enzymes (CAT, POX, SOD, and PPO) and TPC. This strong positive correlation suggests that under these growth conditions, plants responded to potential stress by simultaneously increasing their production of antioxidant enzymes and accumulating phenolic compounds. Phenolic compounds are known to play a role in plant defense mechanisms, and their rise alongside the activities of antioxidant enzymes indicates a coordinated stress response strategy. Interestingly, the disease severity index exhibited a statistically significant negative correlation with TPC and the activities of antioxidant enzymes. This implies that plants with higher levels of these defensive elements experienced lower disease severity. In contrast, PL showed a negative correlation with DSI ($r = -0.816$) (Table 5).

Table 5: Correlation coefficients between disease severity index (DSI), plant length (PL), activities of catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), and polyphenol oxidase (PPO), and the contents of total phenolic (TPC) and total sugar (TS) after 60 days of growth under greenhouse conditions at 25°C

	POX	SOD	PPO	TPC	TP	DSI	PL
CAT	0.928**	0.963**	0.943**	0.889**	0.871**	−0.391	0.345
POX		0.899**	0.928**	0.913**	0.844**	−0.365	0.306
SOD			0.941**	0.884**	0.887**	−0.252	0.319
PPO				0.925**	0.872**	−0.207	0.162

(Continued)

Table 5 (continued)

	POX	SOD	PPO	TPC	TP	DSI	PL
TPC					0.918**	−0.252	0.205
TP						−0.394	0.478*
DSI							−0.816**

Note: **. Correlation is significant at the 0.01 level. *. Correlation is significant at the 0.05 level.

4 Discussion

Metarhizium spp. and *Beauveria* spp., well-established entomopathogenic fungi, play a significant ecological role within agricultural ecosystems by regulating insect populations [59,60]. Their recognized value as biocontrol agents (myco-insecticides) has led to their widespread application in sustainable insect pest management strategies [60,61]. However, recent research suggests these fungi offer even broader potential benefits for plant health. Studies indicated they may function as dual-purpose agents, biostimulants promoting plant growth, and bioprotectants enhancing plant defense mechanisms [62,63]. Notably, research has demonstrated their efficacy in protecting cucumber plants from phytopathogens, including the damaging *R. solani* fungus [63]. This multifaceted ecological role underscores the potential of *Metarhizium* spp. and *Beauveria* spp. as valuable tools for integrated disease management strategies in cucumber production systems [64]. This present study investigated this dual functionality in the context of cucumber cultivation. We examined the potential of *Metarhizium* sp. Me351, *B. bassiana*, and *M. anisopliae* act as biocontrol agents against damping-off and root rot diseases while simultaneously assessing their ability to promote plant growth in cucumber. *B. bassiana* and *M. anisopliae* demonstrated superior efficacy as biological control agents against *R. solani* in cucumber plants. These entomopathogenic fungi not only suppressed the phytopathogen but also improved plant growth and enhanced defense mechanisms.

Extracellular enzyme profiles of *B. bassiana* and *M. anisopliae* isolates revealed a broad spectrum of enzymatic activity. These fungi exhibited positive results for β-1,3-glucanase, pectinase, catalase, protease, cellulase, chitinase, amylase, and lipase enzymes. Existing research suggests these enzymes play a crucial role in degrading the cell walls of various hosts, including fungi, nematodes, and insects [65]. Additionally, they may facilitate nutrient acquisition by utilizing host proteins [65]. The activity of these enzymes is hypothesized to contribute to the inhibition of pathogen growth through mechanisms such as fungal cell wall degradation and limiting nutrient availability [65–67]. Notably, *Metarhizium* sp. Me351 displayed a more selective enzyme profile, lacking protease, chitinase, amylase, and lipase activity. This observed variation in enzyme activity profiles might be linked to the previously reported differences in biocontrol efficacy observed among various fungal isolates [60,65,66].

B. bassiana and *Metarhizium* spp. add another mechanism to their biocontrol arsenal through their ability to produce HCN. Plant growth-promoting fungi (PGPF) are recognized for producing low levels of HCN, which is believed to offer a unique advantage in biocontrol and plant growth promotion [9,68,69]. By maintaining low HCN levels, these PGPF strains may prevent the targeted fungi from developing resistance, ensuring the effectiveness of other antifungal metabolites they produce. This combined action enhances the biocontrol activity of the PGPF strain [9,69]. Furthermore, HCN exhibits antifungal properties by inhibiting the fungal electron transport system. These responses may improve plant tolerance to various environmental stresses, including abiotic and biotic stresses. Ultimately, this translates to improved plant growth and overall health [70,71].

Evaluation of the three fungal isolates revealed a positive capacity for IAA production. The ability of diverse soil microorganisms to synthesize this plant growth hormone, particularly auxin, significantly

influences plant growth and development [72–74]. IAA acts as a regulator in stimulating root elongation and branching, ultimately leading to an increased root surface area. This enhanced root system facilitates improved nutrient and water uptake from the surrounding soil [75,76]. Furthermore, research suggests that IAA plays a role in influencing the biosynthesis of diverse secondary metabolites within plants. These metabolites may contribute to plant resistance against fungal diseases and strengthen their defense responses [59].

Evaluation of cucumber seed germination revealed significant biocontrol efficacy of *B. bassiana* and *M. anisopliae* against *R. solani*. This conclusion is supported by the observed substantial reduction in DSI following treatment with these fungal isolates. Notably, the calculated DSI values categorized these treatments as "highly effective," indicating a statistically significant decrease in disease symptoms compared to the positive control group. The percentage of infected seeds followed a similar pattern, with *B. bassiana* and *M. anisopliae* treatments demonstrating the lowest infection rates. Additionally, the fungal treatments positively impacted germination metrics, including parameters like germination rate and seedling vigor, particularly *B. bassiana* and *M. anisopliae*. These findings suggested that these fungi may promote seed germination and enhance seedling vigor in cucumber plants. This observed growth promotion could be attributed to the enzyme activities and plant growth-promoting traits documented for these fungal isolates [32,63,77].

B. bassiana and *M. anisopliae* have been explored for their potential application in controlling plant pathogenic fungi. Evidence suggests their efficacy against a diverse range of plant pathogens, including *R. solani*, *Pythium myriotylum*, *Sphaerotheca fuliginea*, *Botrytis cinerea*, *F. oxysporum*, *Colletotrichium* spp., *Phytophthora* spp., and *Plasmopara viticola* [37,60,77–85]. Furthermore, entomopathogenic fungi, such as *Metarhizium* spp. and *Beauveria* spp., are known to produce protease enzymes, which play a significant role in their ability to control insect pests. References [37,60,77–85] showed that a similar mechanism might be at play in the observed inhibition of plant pathogenic fungi. Previous studies support this hypothesis, demonstrating a significant increase in healthy seedlings when seeds are cultivated in soil inoculated with *Metarhizium* spp. and contaminated with the plant pathogen *Pythium aphanidermatum*, compared to seedlings grown solely in pathogen-contaminated soil [64]. The observed increase in healthy seedlings is attributed to the potential inhibition of *P. aphanidermatum* by the co-inoculated entomopathogenic fungus [37,77,84–86].

Studies investigating the effect of entomopathogenic fungi on seed germination have yielded promising results, comparatively in chili [87] a nearly 10% increase of seed germination was observed when inoculated with *B. bassiana* and *M. anisopliae* compared to the control groups. Similarly, Reference [88] reported a significant enhancement in corn seed germination following *B. bassiana* inoculation, with inoculated plants exhibiting an 89% germination rate compared to the 77% observed in the control group. Reference [89] further demonstrated that seed treatment with *B. bassiana* not only increased germination rates but also accelerated and synchronized germination compared to untreated seeds. Soaking chili seeds in *B. bassiana* suspensions for 9–12 h resulted in the highest germination rates compared to shorter soaking durations [89]. Reference [90] investigated the influence of entomopathogenic fungi strains on *Vicia faba* plant growth. Their study revealed that inoculation with *M. brunneum* and *B. bassiana* significantly enhanced growth parameters, including seedling emergence, plant height, number of leaf pairs, and fresh root weight. Furthermore, extending the seed treatment duration with these fungi resulted in a positive effect on plant growth, as measured by plant height, number of leaf pairs, fresh shoot weight, and fresh root weight. This finding suggests that increased seed treatment duration may promote a higher plant colonization rate by the beneficial fungi, ultimately leading to enhanced growth responses [90]. Reference [91] reported that endophytic strains of *B. bassiana* and *M. brunneum* improved iron availability, chlorophyll content, root length, and fine root abundance in sorghum. Further studies by [30] and [88] demonstrated enhanced plant growth parameters, including stem height, root length, shoot and root

weight, and yield in wheat and soybean, respectively, when inoculated with *B. bassiana*. Furthermore, research by [92] and [93] suggested that spraying maize seeds with *B. bassiana* and *M. anisopliae* can promote plant growth, evidenced by increased plant height, leaf number, and cob development.

Research suggests that these entomopathogenic fungi may exert their growth-promoting effects through direct and indirect pathways. One potential mechanism involves the production of secondary metabolites by the fungi. These metabolites may include phytohormones that directly stimulate seed germination and root formation in plants. Enhanced root development can ultimately lead to increased nutrient uptake by the plant. Supporting this concept, studies by [94,95], and [96] have shown that *Metarhizium* spp. and *Beauveria* spp. can invade plant root tissues, triggering the formation of root hairs and lateral roots. This increased root development can significantly enhance the plant's ability to acquire nutrients from the surrounding soil. Furthermore, recent research revealed that *Metarhizium* spp. and *Beauveria* spp. may play an indirect role in promoting plant growth by acting as facilitators for nutrient transfer. These entomopathogenic fungi may function as bridges, facilitating the movement of nutrients from the rhizosphere to the plant roots, as evidenced by the work of [64] and [97]. These combined findings provide a compelling framework for understanding the multifaceted mechanisms by which *Metarhizium* spp. and *Beauveria* spp. can promote plant growth [64].

This study examined the antagonistic potential of two entomopathogenic fungi, *B. bassiana* and *M. anisopliae*, against the plant pathogenic fungus *R. solani* under laboratory conditions. Both fungal species significantly inhibited the radial and mycelial growth of *R. solani*, with *B. bassiana* demonstrating a superior inhibitory effect. These results corroborate previous findings by [27,98], and [63] who reported similar suppression of *R. solani* by *B. bassiana*. However, the biocontrol efficacy of *M. anisopliae* may vary depending on the specific strain employed. While this study focused on growth inhibition, it is important to acknowledge the broader biocontrol potential of *M. anisopliae* as evidenced by previous research. References [36,60,66], and [99] reported that *M. anisopliae* strains can additionally suppress the formation and germination of sclerotia and conidia in *R. solani*, suggesting a multifaceted mode of action. The antagonistic interaction between *B. bassiana* and *M. anisopliae* with *R. solani* involves the production of secondary metabolites. These diffusible compounds function as antibiosis agents, documented by [27,36,60,63,66,98], and [99]. The formation of inhibition zones and potential mycelial collapse of *R. solani* can occur before physical contact with the antagonistic fungi. Studies by [27,63,66,100], and [101] identified specific secondary metabolites produced by *B. bassiana* and *M. anisopliae*, including alkaloids, non-peptide pigments, cyclodepsipeptides, and cyclopeptides. These metabolites possess a range of bioactivities, including insecticidal, antimicrobial, and antioxidant properties. In another interaction mode, physical proximity between *R. solani* and entomopathogenic fungi can induce the formation of a distinct deep brown mycelial barrier at the interface. This phenomenon, observed in studies of [36] and [99] is accompanied by aging, browning of *R. solani* mycelia, and increased sclerotial production. The release of beauvericin, a secondary metabolite known to inhibit cell proliferation and induce apoptosis through the mitochondrial pathway, is a potential mechanism for these observed effects. Future research could explore the detailed impact of different secondary metabolites on *R. solani* and their contribution to the overall biocontrol efficacy [102,103].

The current study demonstrated the efficacy of *B. bassiana* and *M. anisopliae*, possessing antimicrobial properties, in mitigating damping-off and root rot diseases in cucumber when applied as a seedling root dip. Entomopathogenic fungi have been shown to reduce plant damage caused by various phytopathogens, including *R. solani* [26,63,64,76,79,85,104]. Pre-planting treatment with *Metarhizium* spp. and *B. bassiana* decreased *Rhizoctonia* disease symptoms, including damage to sprouts, stems, stolons, and sclerotium formation on tubers throughout the growing season [27]. Although *Metarhizium* spp. exhibited greater *in vitro* activity against *R. solani*, field studies demonstrated superior *Rhizoctonia* disease suppression by *B. bassiana* on sprouts, stems, and stolons, while both fungi equally reduced sclerotium

index on tubers [27]. Endophytic colonization of tomato and cotton by *B. bassiana* following treatment significantly reduced damping-off disease caused by *R. solani*, as reported in studies by [105] and [106]. Reference [63] reported a reduction in sheath blight disease incidence following *B. bassiana* application. Additionally, Reference [107] observed a decreased disease incidence percentage in plants treated with *Metarhizium* sp. and *R. solani* compared to *R. solani* treatment alone. A 58.33% reduction in disease incidence was noted in *Metarhizium* spp.-treated plants at 15 DAI relative to *R. solani*-inoculated controls. Furthermore, the suppressive effects of *Metarhizium* sp. against plant pathogens have been documented in other studies. For example, *Metarhizium* sp. inhibited *Cochliobolus heterostrophus* in maize, and *Fusarium solani* f. sp. *phaseoli* was suppressed in haricot beans through soil application of this species [108,109]. These differential effects may be attributed to distinct colonization patterns, with *Metarhizium* spp. primarily inhabiting the rhizosphere and root tissues, and *Beauveria* spp. exhibiting a broader host range, colonizing roots, stems, and leaves [27,76,110,111]. The biocontrol efficacy of *B. bassiana* and *Metarhizium* spp. is attributed to multiple mechanisms, including antagonistic interactions and direct suppression of plant pathogens via mycoparasitism, competition, and antibiosis, as well as indirect effects through endophytic colonization and induction of systemic resistance [30,31,34,37].

Application of *B. bassiana* strain B2 resulted in effective management of rice sheath blight, associated with increased accumulation of defense enzymes, including polyphenol oxidase, peroxidase, chitinase, and lipoxygenase [112]. Reference [113] identified biocontrol mechanisms underlying *B. bassiana* strain TS12 management of *R. solani*-induced tomato damping-off, involving induced systemic resistance through increased accumulation of defense enzymes such as chitinases, peroxidases, and phenyl ammonia lyase and phenolic compounds [113]. *Metarhizium* spp. treatment reduced H_2O_2 and MDA levels by 5.21% and 14.96%, respectively, in pathogen-inoculated plants by enhancing antioxidant enzyme activities (ascorbate peroxidase, glutathione S-transferase, peroxidase, and catalase) [107]. Furthermore, *Metarhizium* spp. increased secondary metabolites, proline, carbohydrates, and soluble sugars in okra, promoting improved osmotic adjustment against diseases. Increased antioxidant enzyme activity mitigates oxidative damage and restores photosynthetic imbalance caused by pathogen-induced lesions [114]. Reference [27] reported increased peroxidase activity four weeks post-planting following treatment with *Metarhizium* sp. and *B. bassiana*, suggesting that soil and rhizosphere colonization of potatoes enhanced root peroxidase activity. Activation of plant-protective enzymes (chitinase, polyphenol oxidase, and peroxidase) has been documented in plant-entomopathogenic fungi interactions [60,115]. Independent endophytic colonization with entomopathogenic fungi can induce plant resistance to phytopathogens and increase protective compound activity [27,38].

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