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Bio-Control Potential of Trichoderma harzianum and Beauveria bassiana against Alternaria solani in Tomatoes (Solanum lycopersicum)

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Abstract

In recent years, there has been a growing demand for sustainable and environmentally friendly methods to control pests and diseases in agriculture. This study focused on exploring the potential of Trichoderma harzianum and Beauveria bassiana, both isolated from Moiben-Kenya, as biological agents for controlling Alternaria solani, a tomato pathogen. In-vitro antagonism tests were conducted using dual culture technique and the impact of culture filtrates were also examined. Potency of volatile organic compounds was evaluated using the inverted plate technique. In dual culture, T. harzianum reduced the growth of A. solani by 48.7% while the culture filtrate did not show any effect. The inhibitory effect of B. bassiana culture filtrates was dose-dependent, with a 5% concentration achieving 8% inhibition, a 10% concentration registering 33% inhibition, and a 15% concentration achieving 39% inhibition. Volatile organic compounds released by T. harzianum inhibited A. solani colonies by up to 50%, while B. bassiana reached a maximum inhibition of 30%. The biocontrol agents were able to exert varying levels of biocontrol towards the test pathogen. It is recommended that in vivo studies be done to assess the application of the biocontrol agents under study in field conditions.

Keywords: Bio-Control, *Trichoderma harzianum*, *Beauveria bassiana*, *Alternaria solani*, Tomatoes

INTRODUCTION

Tomatoes (Solanum lycopersicum) are among the most widely cultivated and economically important vegetable crops worldwide. Despite being a perennial crop, it is grown annually for maximum economic benefits (ChitraMani & Kumar, 2020). Tomatoes are consumed in numerous forms; both fresh and cooked and are liked due to their wide range of colours, shapes, sizes, and flavours (Zörb *et al*., 2020). However, they are highly susceptible to a range of diseases, and early blight caused by *Alternaria solani* is one of the most destructive (Jones *et al*., 2016). Diseases are a major challenge in production of tomatoes, resulting in low yields therefore there is need for management of pathogens of tomato crops (Mulugeta & Selvara, 2013). *A. solani* infects various plant parts, including leaves, stems, and fruits, resulting in significant yield losses and reduced fruit quality.

Traditional control measures against this pathogen rely heavily on chemical fungicides, which not only pose risks to human health and the environment but also contribute to the development of fungicide-resistant strains (Moghaddam *et al*., 2019). As a result, there is a growing interest in exploring alternative approaches such as biological control agents (BCAs). The concept of biological control has gained momentum as a sustainable alternative to chemical control methods (Shanker *et al*., 2011). *Trichoderma harzianum* and *Beauveria bassiana* are two prominent BCAs that have demonstrated promising potential in controlling various plant pathogens (Tall & Meyling, 2018). *T. harzianum* is a mycoparasitic fungus widely recognized for its ability to establish a mutualistic association with plants and act as a bio-control agent against various fungal pathogens (Pusztahelyi *et al*., 2015). *T. harzianum* exhibits multiple mechanisms of action, including mycoparasitism, antibiosis, and induction of systemic resistance in plants (Akladious & Abbas, 2012). These attributes make it a promising candidate for combating *A. solani* infections. *Beauveria bassiana*, is an

entomopathogenic fungus that has also shown significant biological control potential against plant pathogens (Lopez & Sword, 2015). *B. bassiana* produces a plethora of bioactive secondary metabolites, including insecticidal compounds and extracellular enzymes, which exhibit antifungal activities against a broad spectrum of plant pathogens (Wang & Zheng, 2012). Recent studies have reported its efficacy in controlling *Alternaria spp*., suggesting its potential as a bio-control agent for managing early blight disease in tomatoes.

This aim of this research is to investigate the effectiveness of *B. bassiana* and *T. harzianum* in reducing the growth of *A. solani* colonies *in vitro*.

METHODOLOGY

The study area was in Soy constituency (0o 33' 00'' N, 35o 44' 00'' E), Uasin Gishu county, Kenya. In this region, tomato farming is done throughout the year relying on the irrigation from River Moiben during the dry seasons.

Diseased tomato leaves and fruits showing the characteristic symptoms of early and late blight were collected from the field. The samples were separately placed in biodegradable paper bags and transported to the University of Eldoret Biotechnology laboratory. A scanning light microscope was used to observe the specimens in order to study the symptoms of pathogens causing early blight and late blight (Swapan & Chakra, 2012).

The infected tissues from tomato leaves and fruits were surface sterilized using 1% sodium hypochlorite for 30 seconds, then washed three times using sterile distilled water. The infected tissue was inoculated on PDA supplemented with streptomycin and incubated for 7 days at 25oC. The resulting pure cultures of *A. solani* were sub-cultured on pure PDA. Identification of the pathogen was done using cultural and morphological characteristics with the help of published fungal Keys (Dugan 2006). Pure colonies were preserved by transferring the tip of the mycelia into the PDA slants which were kept at 4oC as stock cultures (Swapan & Chakra, 2012).

Trichoderma harzianum was isolated from the rhizosphere of tomatoes in the study area. One gram of soil was added to 0.1% Tween 80 in a bottle, shaken and allowed to rest for 10 minutes. Serial dilutions were performed up to the tenth dilution, from which, 100 microliters were pipetted and spread on *Trichoderma harzianum* selective medium (THSM) while supplemented with streptomycin. This was then incubated at 28oC for 7 days. Sub-culturing of colonies of interest was done on Sabouraud Dextrose Agar (SDA) and later on Potato Dextrose Agar (PDA) media in preparation for subsequent use. Characterization was done by colony observation and spore was observed under the microscope. Greenish white colony color was a preliminary identification of the fungus. Observation of the spores borne on highly branched conidiophore and flask shaped phialide confirmed the identity of the isolates as *Trichoderma harzianum.*

Beauveria bassiana was isolated from dead insects (Mwamburi, 2016) showing signs of infection by entomopathogenic fungi. The insects were obtained from the study area was surface sterilized by use of 1% Sodium hypochlorite (NaOCl) solution then rinsed with sterile distilled water. These were then put on Peptone Glucose Agar (PGA) medium containing Streptomycin and Tetracycline. The plate was then incubated at 28oC for 7 days. After which, colonies of interest were sub-cultured on PDA to obtain a pure colony for subsequent use (Owney *et al*., 2010). Characterization was done morphologically and microscope observation of the spores. The typical white cottony *B. bassiana* colonies were sought for in the isolates. Microscope observation of rachis and the typical conidiogenous cells confirmed the identity of the isolates as *B. bassiana*.

The antagonistic effect of *T. harzianum* and *B. bassiana against* A. solani in vitro was evaluated using the dual culture technique, use of non-volatile and volatile compounds. Discs of 5 mm- diameter from the bio-control agents were

obtained from actively growing edges of 5-days old colonies were separately inoculated on PDA medium 1cm from the edge of the Petri dish. At the centre of each of the petridishes *A. solani* was inoculated. Control plates had *A. solani* without the antagonists. There were three replications in each set. Incubation of the plates were done at 25oC for 7 days. The plates were examined and radial growth of the pathogen towards the antagonist was measured for 7 days so as to compare with those of the controls (Suprapta, 2012). The radial growth (r) towards the antagonist and radial growth in the absence of the antagonist (R) in control plates were taken for 7 days. The radius of inhibition was then obtained by the formula R-r (Makumba, 2016).

For the non-volatile compounds (culture filtrate), the antagonists were grown in potato dextrose broth at 25ºC with intermittent shaking at 150 rpm. The culture filtrate for each were collected after 10 days and filtered first by use of sterile filter papers and later by nitrocellulose filter. The sterilized filtrate was amended in PDA to make 5, 10 and 20% concentration in petriplates. The solidified agar plates in triplicates were inoculated at the centre with 5 mm diameter mycelial disc of *A. solani* and incubated at 25oC for 7 days. Three plates each without filtrate for each set up served as controls. The colony diameter for each treatment was recorded and compared with the controls to obtain the diameter of inhibition (Ajith & Lakshmidevi., 2010).

The effect of volatile compounds from the antagonists on the growth of *A. solani* was determined using inverted plate technique. A petri plate containing PDA was inoculated at the centre with a 5mm disc of actively growing *A. solani* colony. Another plate was inoculated at the centre with 5mm disc of *T. harzianum*. The plates were placed facing each other and sealed with parafilm. They were then incubated at 25oC for 7 days. The same procedure was repeated for *B. bassiana*. Petri dishes containing PDA were similarly inoculated with *A. solani* and un-inoculated PDA plates inverted over and sealed were used as controls. The colony diameter of the pathogen was taken for 7 days (Ajith & Lakshmidevi, 2010).

RESULTS AND DISCUSSION

The colony morphology of *Alternaria solani* was effuse grey in colour, had curved margin, umbonate elevation, cotton felt surface and produced dark red pigmentation that colored the medium (figure 1). When viewed under a microscope at 400X, conidiophores were brown, thick walled, septate and straight (plate 4.5b). Conidia were club-shaped and occurred singly and some in groups.

Trichoderma harzianum

The colony morphology was whitish green forming concentric rings of green conidial production. The green colour was denser in the center and towards the margin. Under a light microscope at magnification of 400×, soft fluffy mycelia, green ring like conidia and highly branched conidiophore and flask-shaped phialide were observed (figure 2).

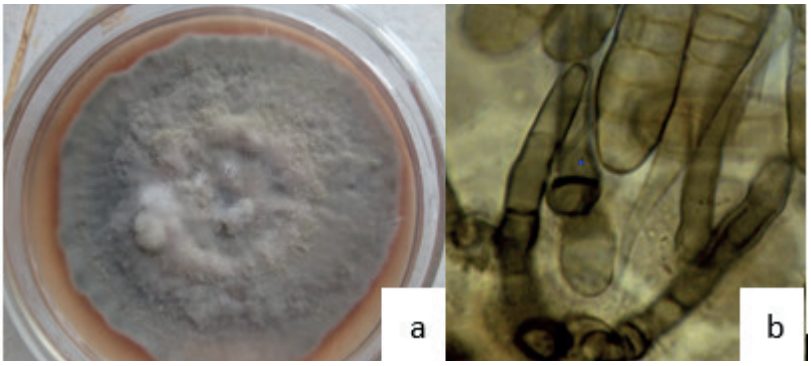

Figure 1. *A. solani* colony (7 days old) on PDA. Note the dark red pigmentation and the effuse grey mycelium. b) A micrograph of *A. solani* under the light microscope at 400× showing brown, thick walled and septate (shown by the arrow) conidiophores with club shaped conidia.

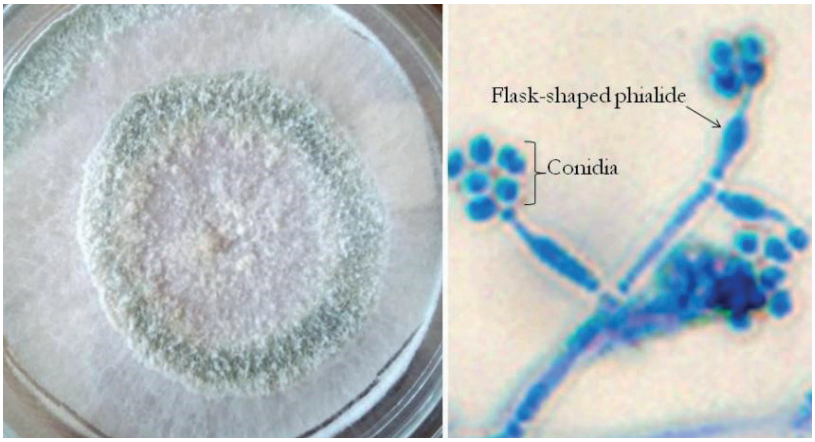

Figure 2. *Trichoderma harzianum* colony (7 days old) on PDA. Note the concentric rings with green coloration (conidia) and the white fluffy mycelium. b) A micrograph of *T. harzianum* under the light microscope at 400× showing a clump of conidia at the tip of a flask-shaped phialide.

Beauveria bassiana

The colony of *B. bassiana* isolate was white in colour, densely wooly and had raised aerial hyphae (figure 3). Cells that were short and ovoid were seen when the slide was viewed under a microscope at 400×. The cells were narrow at the apex forming an extension called rachis. There was a large number of conidiogenous cells.

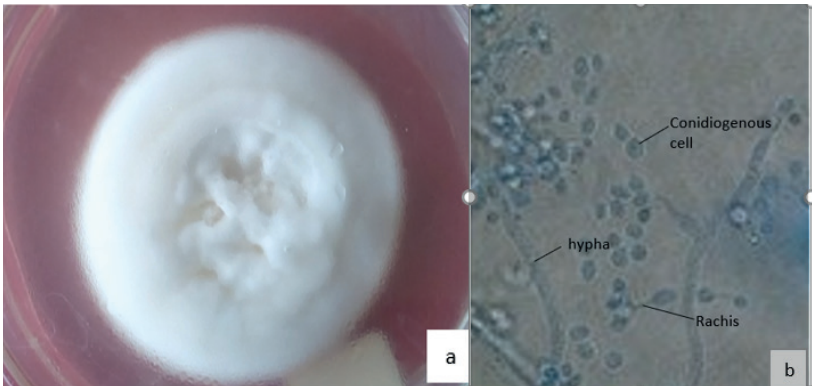

Figure 3. *Beauveria bassiana* colony (10 days old) on PDA. Note the densely cottony mycelium with raised aerial hyphae. b) A micrograph of *B. bassiana* under the light microscope at 400× showing ovoid conidiogenous cells and the rachis.

In vitro tests for antagonism

Dual culture

Trichoderma harzianum inhibited the growth of *A. solani* by 48.7%. Radial lengths were measured from the point at which the pathogen was inoculated towards the antagonist while the control radius was measured from the point of inoculation of the pathogen toward the centre. Fungal colonies growing together with *T. harzianum* attained diameters of approximately 21 mm while the control reached an approximate diameter of 40 mm in 7 days. *T. harzianum* had a faster growing rate compared to *A. solani*. For this reason mainly, *A. solani* was subdued. Fast growth rate endows any organism with colonizing advantage in any ecosystem (Jeyaseelan, 2014). The main mode of action for *T. harzianum* is hyperparasitism. Majorly, mycoparasitism is involved when there is physical interhyphal contact between two fungi (Mukherjee *et al*., 2013). As such a fungus that feeds on another does not produce any chemicals that would inhibit the prey from approaching as close as possible (Kucuk & Kivanc, 2004). Any inhibitory compounds are produced after the victim has been parasitized to further subdue it. Hence, as evidenced in the current study, antagonism by dual culture was not characterized by presence of any zone of inhibition.

The biocontrol fungus *B. bassiana* did not have any apparent impact on the growth of *A. solani*. *Beauveria bassiana* has more reputation as an entomopathogen. However, its biofungicide properties is evident especially with root pathogens like *Fusarium spp* (Kichaoui *et al*., 2017). Further, as an endophyte, it triggers the ability of crops to resist diseases (Sinno *et al*., 2021). However, the evidence provided by this study shows that limited biocontrol abilities towards *A. solani* by dual culture.

Diffusible metabolites

Antagonistic activities of *T. harzianum* diffusible metabolites

Experimentation involved the use of plates with PDA amended with 5%, 10%, and 15% *T. harzianum* culture filtrate. There is very minimal antifungal action of the diffusible metabolites from *T. harzianum* (Figures 5). Colony diameters from the different dosage treatments are broadly similar. This corroborates the earlier argument that a mycoparasite like

T. harzianum would not inhibit the growth of its potential prey through production of inhibitory compounds (Kucuk & Kivanc, 2004). However, strong inhibitions towards some soil bourne pathogens have been observed. Kumar *et al*. (2019) showed the potent nature of the diffusible metabolites towards *Fusarium spp*. this could allude to its adaptive advantage, both occupy the soil and the rhizosphere for that matter. The apparent limited activity observed in the current study could be due to challenging it with a foliar pathogen rather than a root pathogen.

Analysis of variance further shows that the differences in the colony diameter of the colonies in the sixth day ($P = 0.3299$) were not significantly different. Therefore, the diffusible metabolites obtained from the *T. harzianum* culture filtrate do not have any effect on the growth of *A. solani* colonies.

Antagonistic activities of Beauveria bassiana diffusible metabolites

The largest diameter is by the colonies in the control plate with 36mm diameter followed by the 5% v/v (33.33mm) treated plate (8% inhibition). This difference was statistically significant ($P < 0.01$). The 10% concentration on the other hand, recorded a diameter of 24.67mm ((33% inhibition) and the 15% concentration resulted in the smallest diameter of 22mm (15% inhibition). The *B. bassiana* extracts are therefore showing dose dependent antifungal activity. Indeed, apart from the insecticidal activity, *B. bassiana* has the ability to produce bioactive substances that inhibit the growth of other fungi (Narayanasamy, 2013). The major metabolites obtained from *B. bassiana* are oxalic acid, bassianin, assianolides, beauvericin, oosporein, and tenellin (Hernández *et al*., 2020). Of these metabolites, oosporein, tenelin and bassianin are known active antimicrobials (Mascarin & Jaronski, 2016). Production of such diffusible metabolites explains the dose dependent inhibitions observed here.

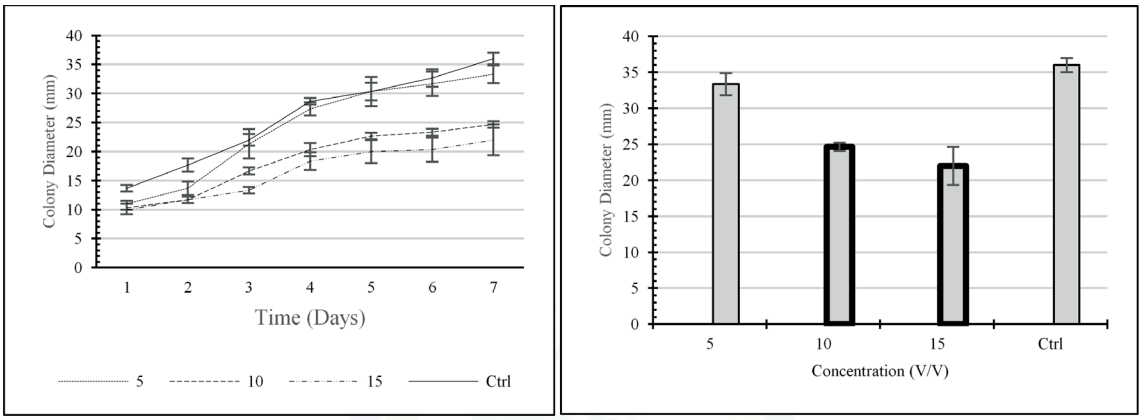

Figure 6. Lines and Bar graphs of growth inhibition of *A. solani* by *B. bassiana* non-volatile metabolites. Dose dependent inhibition is evident.

Antifungal Activity by Volatile compounds

Antifungal activities of T. harzianum Volatile compounds

Antibiosis is another mode of antimicrobial action of *T. harzianum* (Harman *et al*., 2004). Daily measurement of diameter revealed that the effect of *T. harzianum* volatiles organic compounds impacted the growth of *A. solani* from the start and the potency increased with time (Figure 6). Subjecting the seventh day recordings to t-test reveals a statistical significant difference ($P = 0.00038$) in the colony diameters of the two treatments. As a means of exerting direct control

to phytopathogens, *T. harzianum* mainly produces organic volatile compounds (Vinale *et al*., 2008). As observed here where the aerial mycelia were inhibited, the same was observed by (Rubio *et al*., 2023). Many experiments have shown that volatiles from *T. harzianum* have been effective against Aspergillus flavus and Pythium ultimum (Raut *et al*., 2014). The volatiles have also shown to induce plant immunity against potential phytopathogens (Erb, 2018). Perhaps this is an evolutionary adaptation. The plant rhizosphere, is also a habitat for *T. harzianum* (Comenjo *et al*., 2016). Mycoparasitism would ensure that it successfully keeps away competitors from the soil, perhaps the release of volatiles helps secure the habitat by discouraging infections in other parts of a plant.

As earlier mentioned *B. bassiana* is an entomopathogen, but also produces some antimicrobials to keep away competitors (Sahab, 2012). In the current study *B. bassiana* inhibited the growth of *A. solani* by 28.9% by the seventh day (Figure 7). For the first three days however, any antifungal activity was not apparent (figure 13). On the third day, the diameter of the colonies exposed to the volatiles averaged at 24.33mm while the controls were 24.67mm. Some form of inhibition was observed from the 4th day to the final seventh day where a plateau in growth was witnessed. The difference in colonies sizes between these days was significant. The main mode of action for *B. bassiana* volatile compounds is collapsing the mycelium of the competing fungus (Culebro *et al*., 2017). The metabolites interact with hyphae of fungal pathogens and collapse the mycelia before direct contact occurs (Bucarei, 2019).

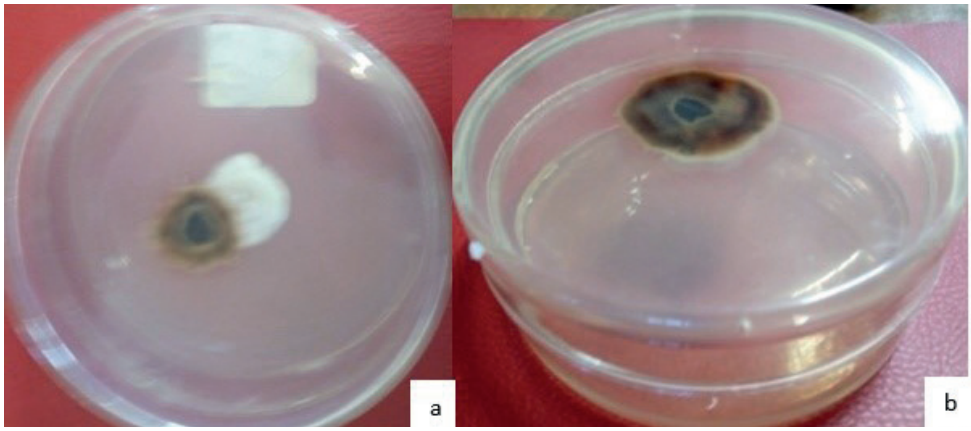

Figure 7. Superimposed plates of *A. solani* and *B. bassiana* on PDA b) Control plate of *A. solani* superimposed against a non-inoculated PDA plate.

CONCLUSIONS AND RECOMMENDATIONS

The biocontrol agents in the current study displayed varying levels of inhibition towards the test pathogen. Volatile compounds produced by the biocontrol agents were effective against *A. solani*. The culture filtrates were effective against *A. solani* and showed dose-dependent potency. In vivo studies will establish the potential of the biocontrol agents to manage *A. solani* in the field.

REFERENCES

Ajith, P. S., & Lakshmidevi, N. (2010). Effect of volatile and non-volatile compounds from Trichoderma spp. against Colletotrichum capsici incitant of anthracnose on bell peppers. Nat Sci, 8(9), 265-269.

Akladious, S. A., & Abbas, S. M. (2012). Application of Trichoderma harziunum T22 as a biofertilizer supporting maize growth. African Journal of Biotechnology, 11(35), 8672-8683.

Amin, M., Mulugeta, N., & Selvaraj, T. (2013). Field Evaluation of New Fungicide, Victory 72 WP for Management of Potato and Tomato Late Blight (P. infestans (Mont) de Bary) in West Shewa Highland, Oromia, Ethiopia. J. Plant Pathol. Microbiol, 4, 192

.Ávila-Hernández, J. G., Carrillo-Inungaray, M. L., De, R., La Cruz-Quiroz, J. E., Paz, D. B., Parra, R., & del Campo, R. (2020). *Beauveria bassiana* secondary metabolites: a review inside their production systems, biosynthesis, and bioactivities. Mexican Journal of Biotechnology, 5(4), 1-33.

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Del-Val, E. K., & Larsen, J. (2016). Ecological functions of Trichoderma spp. and their secondary metabolites in the rhizosphere: interactions with plants. FEMS microbiology ecology, 92(4).

Dugan, F. M. (2006). The identification of fungi: an illustrated introduction with keys, glossary, and guide to literature. American Phytopathological Society (APS Press).

El Kichaoui, A., Elnabris, K., Shafie, A., Fayyad, N., Arafa, M., & El Hindi, M. (2017). Development of *Beauveria bassiana*-based bio-fungicide against Fusarium Wilt pathogens for Capsicum Annuum, a Promising approach toward vital biocontrol industry in Gaza Strip. IUG Journal of Natural Studies.

Erb, M. (2018). Volatiles as inducers and suppressors of plant defense and immunity—origins, specificity, perception and signaling. Current opinion in plant biology, 44, 117-121.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). Trichoderma species—opportunistic, avirulent plant symbionts. Nature reviews microbiology, 2(1), 43-56.

Jones, J. B., Zitter, T.A., Momol, T.M., Miller, S.A. (2016). PART I: Infectious diseases. In Campedium of Tomato diseases and pests, 2nd ed; Jones, J. B., Zitter, T.A., Momol, T.M., Miller, S.A. Eds; The American Phytopathological Society: Saint Paul, MN, USA. Pp. 15-119

Kumar, M., Vipul, K., Meenakshi, R., & Seweta, S. (2019). Effect of volatile and non-volatile compounds of Trichoderma spp. against Fusarium isolates causing chickpea wilt in Punjab. Plant Archiv, 19(1), 159-162.

Kumar, V., Singh, G., & Tyagi, A. (2017). Evaluation of different fungicides against Alternaria leaf blight of tomato (*Alternaria solani*). International Journal of Current, Microbiological and Applied Sciences, 6, 2343–2350.

Lopez, D. C., & Sword, G. A. (2015). The endophytic fungal entomopathogens *Beauveria bassiana* and Purpureocillium lilacinum enhance the growth of cultivated cotton (Gossypium hirsutum) and negatively affect survival of the cotton bollworm (Helicoverpa zea). Biological Control, 89, 53-60.

Makumba, B., Mwamburi, L., & Kiprop, E. (2016). Management of sorghum anthracnose using bio-control agents produced by sorghum rhizobacteria in western Kenya.

Mascarin, G. M., & Jaronski, S. T. (2016). The production and uses of *Beauveria bassiana* as a microbial insecticide. World Journal of Microbiology and Biotechnology, 32, 1-26.

Moghaddam, G. A., Rezayatmand, Z., Esfahani, M. N., & Khozaei, M. (2019). Genetic defense analysis of tomatoes in response to early blight disease, Alternaria alternata. Plant Physiology and Biochemistry, 142, 500–509.

Mukherjee, K. K., Das, D., Samal, A. C., & Santra, S. C. (2013). Isolation and characterization of Arsenic tolerant fungal strains from contaminated sites around urban environment of Kolkata. IOSR. J. Environ. Sci. Toxicol. Food Technol, 7(5), 33-37.

Mwamburi, L.A. 2016. Isolation and Assessment of Stability of Six Formulations of Entomopathogenic *Beauveria bassiana*. In: Glare, T.R. Moran- Diez, M.E (Eds.), Microbial-Based Biopesticides: Methods and Protocols. Series: Methods in Molecular Biology, Springer, Humana Press Inc. USA. Volume 1477 Chapter 4. pp 85-91.

Narayanasamy P. (2013). Characteristics of biological control agents. In: Biological Management of Diseases of Crops, (Hokkanen. H. M. T., ed). Springer. Dordrecht,

Ownley, B. H., Gwinn, K. D., & Vega, F. E. (2010). Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. Bio Control, 55(1), 113-128Países Bajos. https://doi.org/10.1007/978-94-007-6380-7.

Pusztahelyi, T., Holb, I. J., & Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. Frontiers in plant science, 6, 573.

Raut, I., Badea-Doni, M., Calin, M., Oancea, F., Vasilescu, G., Sesan, T. E., & Jecu, L. (2014). Effect of volatile and non-volatile metabolites from Trichoderma spp. against important phytopathogens. Revista de Chimie, 65(11), 1285-1288.

Rubio, M. B., Monti, M. M., Gualtieri, L., Ruocco, M., Hermosa, R., & Monte, E. (2023). *Trichoderma harzianum* Volatile Organic Compounds Regulated by the THCTF1 Transcription Factor Are Involved in Antifungal Activity and Beneficial Plant Responses. Journal of Fungi, 9(6), 654.

Shanker, C., Katti, G., Padmakumari, A. P., Padmavathi, C., & Sampathkumar, M. (2011). Biological control, functional biodiversity and ecosystem services in insect pest management. In Crop Stress and its Management: Perspectives and Strategies (pp. 471-495). Dordrecht: Springer Netherlands.

Sinno, M., Ranesi, M., Di Lelio, I., Iacomino, G., Becchimanzi, A., Barra, E., ... & Woo, S. L. (2021). Selection of endophytic *Beauveria bassiana* as a dual biocontrol agent of tomato pathogens and pests. Pathogens, 10(10), 1242.

Suprapta, D. N. (2012). Potential of microbial antagonists as biocontrol agents against plant fungal pathogens. J ISSAAS, 18(2), 1-8.

Tall, S., & Meyling, N. V. (2018). Probiotics for plants? Growth promotion by the entomopathogenic fungus *Beauveria bassiana* depends on nutrient availability. Microbial ecology, 76(4), 1002-1008.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., ... & Lorito, M. (2008). A novel role for Trichoderma secondary metabolites in the interactions with plants. Physiological and molecular plant pathology, 72(1-3), 80-86.

Wang, J., & Zheng, C. (2012). Characterization of a newly discovered *Beauveria bassiana* isolate to Franklimiella occidentalis Perganda, a non-native invasive species in China. Microbiological research, 167(2), 116-120.

Zörb, C., Piepho, H. P., Zikeli, S., & Horneburg, B. (2020). Heritability and variability of quality parameters of tomatoes in outdoor production. Research, 2020.