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The efficacy of *Trichoderma* spp. and *Bacillus* isolates in the control of chickpea wilt pathogens

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Abstract: Dual experiments were carried out in 2007 at the laboratories of the National Center of Research, to test the antagonistic efficacy of three *Trichoderma spp* and *23 Bacillus* isolates, for the control of chickpea wilt and root- rot pathogens: *Fusarium oxysporum f. sp. ciceris* and *F. solani* adopting CRD. *Trichoderma harzianum* was found highly antagonistic compared to *Trichoderma viride* isolates as it inhibited the mycelial growth of *F. oxysporum f. sp. ciceris* and *F. solani* by 85.29% and 86.21% after 12 days of *in-vitro* incubation, whereas *T. viride* (isolate Tv1) gave an inhibition percentage of 81.88% and 76.64%. Antagonistic hyphae of *T. harzianum* showed parasitic behavior against Fusarium spp. The parasite reached and recognized *F. oxysporum f. sp. ciceris* by coiling around the hyphae of the pathogen and disintegrating the hyphae and spores. Only 17 out of 23 Bacillus isolates from 130 colonies of bacteria screened showed significantly antagonistic properties against wilt pathogens. Only B3, B16, B2, B15and B20 proved to be the most effective among the rest of isolates and were considered strongly antagonistic against *F. oxysporum f. sp. ciceris* and *F. solani in-vitro*, with an inhibition percentage range of 57.57% - 64.65%. The management of Chickpea root/rot wilt complex disease incited by *F. oxysporum f. sp. ciceris* and *F. solani* could be achieved successively by the use of bioagents derived from various fungal and bacterial isolates.

Keywords: Antagonisms, *Bacillus* spp, Biocontrol, *Cicer arietinum* L, *Trichoderma harzianum*, *T. viride*

1. Introduction

Chickpea, *Cicer arietinum* L., is one of the best legumes for human consumption and widely grown in Northern Sudan. *Fusarium oxysporum f. sp. ciceris* and *F. solani* are the wilt and root- rot pathogens causing severe damage wherever this crop is grown [1, 2].The use of bioagents in the control of pests is a result of the change in the public attitude towards the use of chemical pesticides and fumigates [3]. In this respect, *Trichoderma* spp. has been studied as biological control agents against soil-borne plant pathogenic fungi and nematodes. They have been investigated for over 80 years and recently used as biological control agents and their isolates have become commercially available. The mechanisms suggested to be involved in their bio-control are antibiosis, lysis, competition, mycoparasitism and promotion of plant growth [4].

Plant growth promotion by Plant Growth Promoting Rhizobacteria (PGPR) may also be an indirect mechanism of biological control, leading to disease escape when the growth promotion results in shortening the time that a plant is in a susceptible state [5]. It was originally reasoned that endophytic bacteria which could colonize vascular tissues of plants would be potential antagonists of vascular-invading pathogens, such as *F. oxysporum* and *Verticillium* spp. [6]. One of the advantages of using entophytes is that, once inside the host, they are better protected against environmental stress and microbial competition [7]. Strains of Bacillus are among the most common bacteria found to colonies plants endophytically and are plant growth promoters [8]. *Bacillus* spp. has the characteristics of being widely distributed in soils, having high thermal tolerance, showing rapid growth in liquid culture, and readily form resistant spores. Abeysinghe [9] screened four isolated rhizobacteria in dual Petri dishes assay as antagonistic against *F. solani f.sp. phaseoli* and reported that *Bacillus subtilis* CA32 effectively antagonized the pathogen growth by 55.05% mycelium inhibition mean. Harlapur et al.[10] evaluated eight bio-agents under *in-vitro* conditions against *Exserohilum turcicum*, that affect maize

leaf, and among the bio-agents tested *T. harzianum* caused significantly maximum inhibition (65.17%) followed by *T. viride* (56.95%) and *B. subtilis* (49.57%), while *Pseudomonas fluorescens* was found to be the least effective (19.30%).

The objective of this study is to test the antagonistic ability of three *Trichoderma* spp and two *Bacillus* isolates obtained from chickpea growing areas at Northern Sudan against *F. oxysporum f. sp. ciceris* and *F. solani* the causal agents of wilt root rot disease complex. This disease is highly sever at chickpea grown areas, that leads the farmers to alter the crop with other profitable alternative crops. The morphological antagonistic behaviour of *Trichoderma* spp *in- vitro* were also observed.

2. Materials and Methods

2.1. Source of Pathogens

Soil samples were randomly collected at March 2007 from the rhizosphere of chickpea grown on a sick plot at Shambat Research Station Farm (ARC), heavily infected with wilt/ root-rot disease complex incited by *Fusarium* spp and the lesion nematode *Pratylenchus* spp. The sick plot is repeatedly used for chickpea Fusarium wilt resistance screening experiments. The soil was maintained in paper bags and left to dry at room temperature (25-30^{0}C).

2.2. Isolation and Identification of Fusarium spp

Fusarium spp were isolated from air-dried soil samples following the dilution plate method [11]. The samples were thoroughly mixed, and a suspension of 1g (dry weight equivalent) in 9 ml of sterilized distilled water was prepared from each sub-sample. A serial dilution of the soil suspensions was prepared (ten-folds), and inoculated on a Fusarium selective Spezieller Nährstoffarmer Agar media (SNA) that composed of KH2PO4 1.00 g, KNO3 1.00 g, MgSO4.7H2O 0.50 g, KCl 0.50 g, Glucose 0.20 g, Sucrose 0.20 g, Agar 20.00 g, Distilled water 1.00 L, supplemented with 0.05g chloramphenicol as anti-bacterial. And 1-2 pieces of sterile filter paper (Whatman № 1) were placed, approximately 1 cm² on the agar surface to enhance sporulation. The media were allowed to dry for 3 days [15]. Individual colonies resembling *Fusarium* spp were transferred to Potato Dextrose Agar (PDA) media (Unpeeled potato 200g, Dextrose 20g, Agar 20g, Distilled water 1 L) and incubated for 10– 15 days at 25°C in the dark. Production of pigmentation was observed on SNA media. The isolates were maintained on sterilized soil-agar at 4°C and / or PDA medium at 25°C and sub-cultured every three months. Identification of isolates was based on cultural, microscopic characteristics with reference to Leslie and Summerell's Fusarium laboratory manual [12].

2.3. Isolation of Antagonistic Bacteria (SciencePG-Level3-Multiple-Line)

Twenty- three bacteria isolates were used in this study. Thirteen strains were isolated from the rhizosphere of the main chickpea growing areas of Adu Hamad (northern Sudan). Eight grams of soil samples were transferred to a 100 ml beaker and filled with 40ml of sterilized water. The beaker was heated in a water path for 10 minutes at approximately 80^{0}C and agitated during the process. The soil suspension was serially diluted, spread on Nutrient Agar (NA), on Petri plates using a spreader and inoculated at 28^{0}C for 48 h. Five replicated plates were prepared for each dilution (1x10^5 and 1x10^7). Colonies were isolated on the basis of their different visual characteristics. After isolation, all colonies were purified by single colony isolation after triple re-streaking on NA medium. Colonies morphologically resembling *Bacillus* species were subcultured and maintained on NA tubes according to Claus and Berkeley [13]. The bacterial isolates were stored in NA tubes at 4^{0}C and sub-cultured every three months. Active cultures were prepared in NA tubes 48 h before application.

2.4. Isolation of Trichoderma spp and Inoculum Preparation

The fungal antagonists were isolated from soil samples brought from Shambat area (Central Sudan), using dilution plate techniques [11] on Trichoderma selective medium, cultures were incubated for 2 days on SNA medium in the dark, followed by incubation under ambient laboratory conditions of light and temperature (about 23°C). After an incubation period, colonies determined to be *Trichoderma* spp., were purified and identified on the basis of their morphological characters [14]. *Trichoderma* spp. were maintained on PDA media and stored at 4 °C. Two isolates were isolated and identified in this study *Trichoderma harzianum* (Ts) and *T. viride* (Tv1), the other isolate were obtained from Khartoum Crop Protection Department (isolated from northern Sudan soil) and were identified as *T. viride* (Tv2).

The inocula of *Trichoderma* spp were prepared from 8 – 10 days old culture grown on PDA media. Ten (10) ml of sterilized distilled water were added to each Petri dish, and the surface of the culture was scraped with a glass spatula to dislodge the spores. The spore suspension derived from six Petri dishes was transferred to 100 ml sterilized flask. One ml of each isolate was poured into PDA Petri dishes, allowed to dry for 3 days, and incubated at room temperature for one day.

2.5. In-Vitro Antagonistic Tests

The *in-vitro* antagonistic properties of bacteria isolates and *Trichoderma* spp were investigated against *Fusarium* spp. Assay were performed in Petri plates (90 mm) containing 20 ml of PDA, allowed to dry for 3 days. An entire pure culture, 4 mm in diameter obtained from one day old *Trichoderma* spp and 7-10 days old cultures of *F. oxysporum f. sp. ciceris* and *F.solani* were cut using a cork-borer. Pathogens disks were transferred 10 mm from the edge of each Petri dish. Each *Trichoderma* spp was placed 10 mm from the other edge, opposite to the pathogens. The inoculated plates were then incubated upside-down at 25^{0}C, and were observed for

inhibition or otherwise of their growth for 12 days. Each bacterial isolate were spotted with a sterile tooth- stick (four spots per plate), 10 mm from the opposite edge of the Petri dish and opposite to the pathogens [15] and were observed for inhibition for 7 days. The control Petri dishes were inoculated each alone with *F. oxysporum f. sp. ciceris* and *F.solani*. The radial growth of the two pathogens in the control and the treated Petri dishes were measured every 24 h, and the inhibition percentage of the antagonism was calculated according to the following formula:

The percentage inhibition = R1 - R2 / R1 ×100

Where R1 is the value of radial growth of pathogen in control plates and R2 is the radial growth of the pathogen in the treated plates [16].

2.6. Analysis of Data

A completely randomized design (CRD) with five replicates for the effect of Trichoderma spp and three replicates for bacterial effect was adopted in this part of the study. The percentage of inhibition was arc sin transformed and the data means were analyzed according to Duncan multiple range test at $P \leq 0.05$ to test significant differences between treatments [17].

3. Results

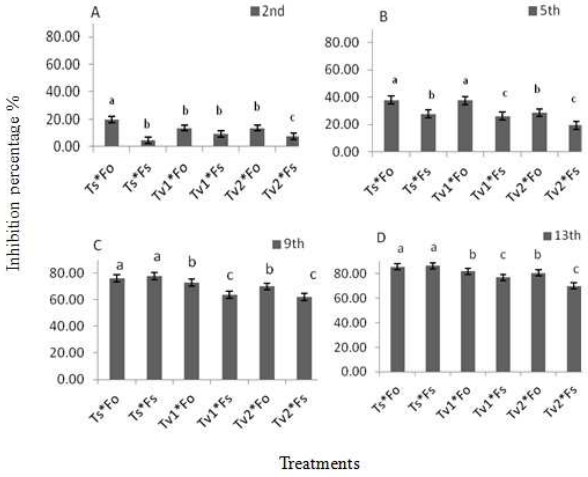

Figure 1. *Mean inhibition percentages of the radial growth of F. oxysporum f.sp. ciceris and F.solani treated with three Trichoderma isolates on PDA media from the 2nd day after inoculation. .Vertical bars represent standard errors. Bars with the same letter are not significantly different at P ≤ 0.05.*

Using the serial dilution isolation technique described above, thirteen isolates were preliminary characterized as member of the genus Bacillus based on its colony morphology, gram-positive reaction, spore forming and the presence of the bacillus-shape under the electron microscope [13]. The fungi isolated from the sick plot (Shambat Research Station Farm) were identified as *F. oxysporum f. sp. ciceris* and *F. solani* according to Leslie and Summerell's Fusarium laboratory manual [12]. Whereas three species of Trichoderma were isolated from Shambat area (Central Sudan), one isolate was identified as *T. harzianum* (Ts), and two were identified as *T.viride* (Tv1 and Tv2) according to [14]

Antagonistic properties of *T.viride* (Tv1 and Tv2) and *T. harzianum* (Ts) were tested against the *F. oxysporum f. sp. ciceris* and *F. solani*, using dual Petri plate method. It appeared from the data presented in Figure 1 that all the antagonistic fungi significantly ($P \leq 0.05$) inhibited the growth of *F. oxysporum f. sp. ciceris* and *F.solani* against the control plates during all days of treatments. *T. harzianum* were found significantly superior in antagonizing the two pathogens than *T. viride* isolates, inhibiting the mycelial growth of *F.oxysporum f. sp. ciceris* and *F. solani* with a range of 19.78% to 85.29% and 4.52% to 86.21% respectively during the incubation days (Fig.1) with no significant difference at the13th day after inoculation. The effect of *T.harzianum* was highly superior from the second day of inoculation recording an inhibition percentage of 19.78% in comparison with the other species. After 5 days of inoculation, *T. harzianum* resulted in inhibiting the two pathogens by 37.93%, 75.90%, 85.29% and 27.83%, 77.70 %, 86.21% during the 5th, 9th and 13th days after inoculation; respectively (Fig.1.A, B, C).

T. viride isolates (Tv1 and Tv2) gave an inhibition percentage of 37.62%, 28.67% and 26.17%, 19.52% after 5 days; respectively (Fig.1A) and 72.79%, 69.84% and 63.42%, 61.80% after the 9th day; respectively (Fig.1B) and 81.88%, 80.29% and 76.64%, 69.67% after the 13th day after inoculation; respectively (Fig.1C).

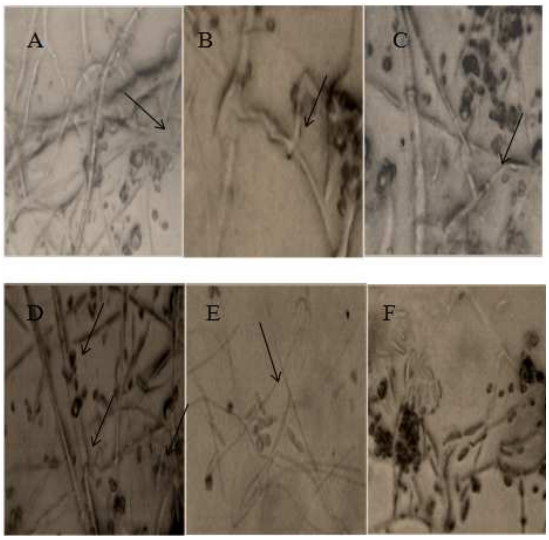

Figure 2. *(A) The parasitic hyphae of T. harzianum reached and recognized the host hyphae F.oxysporum f.sp. ciceris (B) coiling, (C) encircling, (D) penetrating the mycelia and disintegrating the hyphal cell wall, (E) Normal septation of F.oxysporum f.sp. ciceris spores, (F) invaded and disintegrated spores by T. harzianum with no or faint septation as shown by the arrows.*

From day five onward, the inhibition percentage of the mycelial growth of pathogens by *Trichoderma* isolates increased (Fig.1). From day 9 to the end of the experiment *F.oxysporum f. sp. ciceris* and *F. solani* were more sensitive. *T. viride* isolates were never observed to overgrow the tested fungus. Among the two *T. viride* isolates isolate Tv1 was significantly ($P \leq 0.05$) more effective in inhibiting the

mycelial growth of the two pathogens.

Observations of hyphal interaction indicated that antagonistic hyphae of *T. harzianum* showing parasitic behavior against Fusarium spp. The parasite reached and recognized the pathogen (Fig. 2A), by coiling around the hyphae of pathogen and disintegrating them (Fig. 2B and C). Occasionally *T. harzianum* hyphae formed a hook or bunch like structures around the hyphae of the pathogen from where penetration took place (Fig. 2C and D). Hyphae of antagonist either coiled around the hyphae of *F. oxysporum f. sp. ciceris* before penetration (Fig. 2B) or entered directly without the formation of appressorium-like structures suggesting mechanical activity (Fig. 2D). The host hyphae eventually disintegrated (Fig.2E).The spores of *F. oxysporum f. sp. ciceris* was disintegrated shown no or faint septation (Fig. 2F). The antagonistic mycelium of *T. harzianum* overgrew on the mycelium of F. oxysporum f. sp. ciceris after day 10, whereas *T. viride* isolates only arrested the mycelium growth of *F.solani*.

Twenty three bacterial isolates were tested *in- vitro*, out of 130 colonies isolated from the infected soil, for inhibition of mycelium growth of *F.oxysporum f.sp. ciceris* and *F. solani* (Fig. 3). Sixteen isolates suppressed mycelium growth of *F.oxysporum f.sp ciceris* but nine isolates, B3, B16, B2, B15, B22, B20, B11 and B18 were significantly ($P \leq 0.05$) superior to the rest of isolates (Fig. 3A), with an inhibition percentage of 64.65%, 63.95%, 62.60%, 62.60%, 58.22%,57.57% 51.64%, and 50.96%, respectively. Also the mycelial growth of *F. solani* was significantly ($P \leq 0.05$) suppressed by nine isolates, B3, B4, B6, B10, B11, B14, B16, B19, and B22, but the most effective isolates were B16, B3 and B22 (Fig. 3B) with an inhibition percentage of 55.76%, 51.06% and 49.17% respectively.

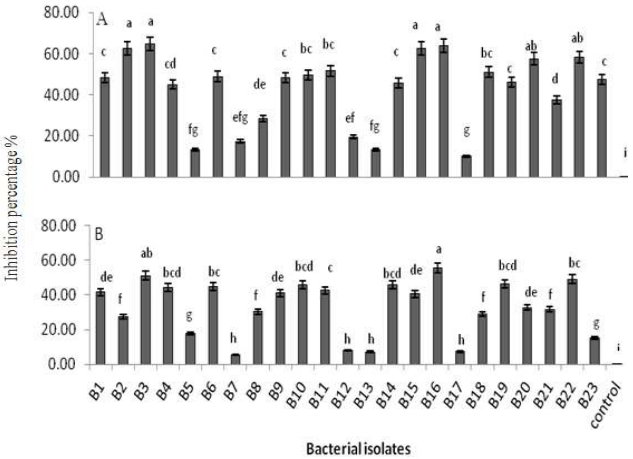

Figure 3. *Mean inhibition percentage of (A). F.oxysporum f.sp. ciceris and (B). F.solani treated with 23 bacterial isolates on PDA media after 7 days of inoculation. Vertical bars represent standard error from the means. Bars with the same letter are not significantly different at P ≤ 0.05.*

These three isolates were considered highly inhibitory against the two pathogens. *F.oxysporum f.sp ciceris* was more sensitive to bacterial isolate than *F. solani* evidenced by the inhibition percentages recorded "Fig. 3". However, for the rest of the bacterial isolates, *F.oxysporum f.sp ciceris* and *F. solani* over grew the bacterial isolates. In the control treatments the phytopathogens completely covered the Petri dishes.

4. Discussion

The results obtained suggested that *Trichoderma* spp. is capable of producing a range of metabolites, which have antifungal activity. *T. harzianum* and *T. viride* significantly ($P \leq 0.05$) inhibited the growth of fungal pathogens and reduce the mycelial growth indicating antagonistic properties against the pathogens. Somasekhara et al. [18] reported that bioagents such as *T. viride*, *T. harzianum* and *T. hamatum* are effective in controlling pigeonpea wilt caused by *F. oxysporum f. sp. udum*.

The minimum growth inhibition percentages of *Fusarium* spps (8.45%-34.43%), incited by *Trichoderma* isolates were at 4 and 5 days after incubation. This may be due to high pathogenic virulence of the pathogens, which resisted the inhibitory action of *Trichoderma* species. From day 6 a gradual increase in the inhibition action was recorded, attaining its maximum of 86.21% at day 13 after inoculation. Overgrowing behaviour of the pathogen was observed when *F. oxysporum f.sp. ciceris* was treated with *T. harzianum*. The *Trichoderma* spps used in this study were found more aggressive antagonisms than that used by Nikam et al. [19] who reported that *T. harzianum* was more effective in inhibiting the mycelial growth of *F. oxysporum f.sp. ciceris* by 83.33% than *T. viride* (76.66%). Production of chitinolytic and glucanolytic enzymes from *T. harzianum* may have direct significance in the parasitism on *F. oxysporum f.sp. ciceris* as these enzymes function by breaking down the polysaccharides, chitin and β-glucan that are responsible for the rigidity of fungal cell walls and interact synergistically to achieve a high level of antifungal activity, thereby destroying cell wall integrity, which could explain the strong antagonistic results of *T. harzianum* [20,21].

It was observed that the antagonisms were expressed as arresting the mycelium growth of *F.solani*. One of possible mechanisms is fungistasis, in which inhibition of germination has been considered a survival mechanism which arrest fungal growth and biologically controls the pathogenic fungi [22]. There could be other reasons for this result; for instance, the increase was favored through antibiotic production by the mature mycelium. Such antibiotics had indeed been reported by workers like Jayaswal et al. [23], Mcloughlin et al. [24] and Montealegre et al. [25] who reported that *Trichoderma* spp. secreted chitinase and B-1, 3 glucanase in supernatants.

The *in vitro* culture of *Fusarium* spp and *Trichoderma* spp in culture media led to a variety of interactions. *Fusarium* spp growth was generally inhibited; the host cell contents disorganized and the hyphae were intensively parasitized by *Trichoderma* spp. similar reactions have been reported on other fungal pathogens [26].

Mycoparasitism behaviour displayed by *Trichoderma* spp. was evidenced by the microscopic observations of the interaction regions between *F.oxysporum f.sp. ciceris* and *T.*

harzianum. The mycelia of *T. harzianum* grew on the surface of the pathogen always coil round their mycelia and later penetrate their cell walls directly without formation of appressorium structures. The mycelia of the pathogen then disintegrate suggesting an enzyme action [27, 28]. Lorito et al. [20], Metcalf and Wilson [29] and Sharon et al. [30], demonstrated the possible role of chitinolytic and/or glucanases enzymes in the biocontrol exhibited by Trichoderma. These enzymes function by breaking down the polysaccharides, chitin, and glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity limiting the growth of the pathogen. A mixture of several enzymes might be necessary for efficient cell wall lysis. *T. viride* and *T. harzianum* were reported by several workers as the best antagonists for growth inhibition of several soil and seed borne plant pathogens [31, 32].

From the 23 bacterial isolates used, only nine isolates were able to antagonize *F. oxysporum f.sp. ciceris* in dual Petri plate assay. Isolate B3, B16, B2, B15, B 22, B20, B11, B18 and B10 significantly inhibit *F. oxysporum f.sp. ciceris* radial growth recording a percentage inhibition range of 64.65% - 50.96%, on the other hand, only three isolates were highly significantly able to inhibit *F.solani* percentage radial growth namely B16, B3 and B22, with a percentage inhibition of 55.77%, 51.05% and 49.17%; respectively. These results were in agreement with that reported by Abeysinge [11]. Some bacteria isolates change the media colour especially near the inhibition zone resulting in mycelial growth retardation. This behavior was observed when *F. oxysporum f.sp. ciceri* was treated with B3 and B16, which assumed to be a result of excretion of inhibitory substances or enzymes. Reduction of fungal growth by certain PGPR and formation of inhibition zones were presumably due to the materials; antifungal substances and/or cell wall degrading enzymes; released by the bacteria into the culture medium [33]. Also, Sarhan et al. [34] and Montealegre et al. [25] pointed that the cell free culture filtrate of *B. subtilis* inhibited the mycelial growth, radial growth, and spore germination and germ-tubes length of *F. oxysporum f.sp. ciceri*. Many strains of *Bacillus* strains have been found to be potential biocontrol agents against fungal pathogens. This antifungal action involves the production of antibiotics, especially within soil microsites [35]. However, it is likely that several mechanisms act in concert to achieve control, including the production of volatiles, which have a significant effect on soil microbiology, including the soil-borne plant pathogens such as *Rhizoctonia solani* and *Pythium ultimum* [36]. The isolates B3 and B16 were the strongest antagonistic isolates against the two pathogens.

The protection exerted by the Trichoderma isolates against the fungal pathogens was pronounced than *Bacillus* isolates conformed by the high mycelial radius growth inhibition percentages, which confirms the results of Harlapur et al. [10].This difference may be due to more than one mode of mechanisms exerted by the *Trichoderma* spp which may have an additive effect in plant protection. Moreover, *Trichoderma* spp is a well-known producer of cell wall-degrading enzymes and antibiotics thus could act synergistically with other mechanisms [37]. *F.oxysporum f.sp ciceris* was more sensitive against fungal and bacterial antagonisms than *F. solani* evidenced by the inhibition percentages recorded.

In conclusion, the present study clearly demonstrated that antagonistic *Bacillus* isolates and *Trichoderma* spp can be used as biological control agents in order to protect chickpea plants from wilt/root-rot pathogens. The combined use of these biocontrol agents and the evaluation of the biological control efficacy under pot trail conditions are underway.

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