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EFFICACY OF THE BIOAGENTS *BACILLUS* ISOLATES AND *TRICHODERMA* SPP. IN THE CONTROL OF WILT/ROOT-ROT DISEASE IN CHICKPEA

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ABSTRACT

Purpose: The aim of this study was to investigate the ability of four microorganisms to reduce disease infection of root lesion nematode (*Pratylenchus* spp) and *Fusarium* spp., the causal agent of wilt/root-rot disease complex in chickpea.

Design/methodology/approach: A pot experiment was conducted for three consecutive winter seasons. A completely randomised block design with five replicates was adopted. Two *Bacillus* isolates and *Trichoderma harzianum* and *T.viride* and their combinations were applied to infected soil. The effect on plant growth parameters, disease incidence and severity, root necrosis, weight of shoot and root, nematode population density and reproductive index were assessed.

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Findings: The applications significantly ($P \leq 0.05$) reduced the wilt/root-rot diseases complex. The reduction was attributed to the decline of the population density of nematodes in the soil and root, and the suppression of the disease complex compared to controls, represented by reproductive index and the Disease Intensity Index (DII). Combinations were better than individual inoculation. The use of *Bacillus* isolate B3and *T. harzianum* increased the number of flowers by 88.34%. Reduction in the severity of root necrosis was in the range of 2.22–5.55 within a scale of 1–10. These findings indicate the significance of utilising local bioagents for control of wilt/root-rot disease complex in chickpea plants.

Original/Value: The microorganisms used in this study are indigenous.

Keywords: *Cicer arietinum* L.; *Pratylenchus* spp.; *Fusarium oxysporum f.sp. ciceris*; *Bacillus* spp.; *Trichoderma harzianum*; *T. viride*; *Tv*; Biocontrol.

INTRODUCTION

Fusarium wilt and root-rot disease, caused by *Fusarium oxysporum* Schlechtend.Fr. *f. sp. ciceris* (Padwick) Matuo & K. Sato and *F.solani* is a major constraint to production wherever chickpea (*Cicer arietinum* L.) is grown (Haware, 1990). Many species of root-lesion nematodes (*Pratylenchus* spp.) are very important plant parasites that affect chickpea production. The presence of these pathogens in chickpea fields may interact synergistically, causing more damage and a higher level of infection than would result from either pathogen alone (Castillo et al., 1998).

Management of disease complexes appears to be less straight forward than one might anticipate. Apart from chemicals, various workers suggested other control measures to replace the highly toxic and potentially polluting chemicals used to control plant parasitic nematodes and fungi, preferably with biological control agents and botanicals (Oostendrop and Sikora, 1989). Results from different studies showed that several strains of Trichoderma had a significant reducing effect on plant diseases caused by pathogens such as *Rhizoctonia solani*, *Sclerotium rolfsii*, *Phythium aphanidermatium*, *F. oxysporum*, *F. culmorum* and *Gaeumannomyces graminis* var. *tritici* under greenhouse and field conditions (Kucuk and Kivanc, 2003). The mechanisms suggested as being involved in bio-control by these fungi are antibiosis, lysis, competition, mycoparasitism and promotion of plant growth (Odebode, 2006).

Soil treatment with *T.harzianum* resulted in greater growth, increased transpiration and reduced wilting index of *F. oxysporum.* f.sp. *ciceris* (Siddiqui and Singh, 2004). A reduction in egg production in the root knot nematode, *Meliodogyne arenaria* were reported following soil treatments with *T.harzianum* (T.22) and *T.koningii* (T.8) (Haseeb et al., 2005). In naturally infected fields with *Pratylenchus thornei* combined seed treatments with *T. viride* and *Pseudomonas fluorescens* increased chickpea yield up to 29.50% and the final nematode population was reduced by 22.30% (Dwivedi and Johri, 2003). Whereas, simultaneous application of *P. fluorescens* plus *T.harzianum* and *T.viride* separately decreased *P. thornei* population in soil and root by 73% and 61%, respectively (Bhatti and Kraft, 1992).

Besides fungi, Plant-Growth Promoting Rhizobacteria (PGPR) have also been used for biological control of soil borne pathogens. They have the ability to improve plant growth by colonising the root system and suppressing harmful rhizosphere microorganisms (Akhtar and Siddiqui, 2009). These provide biocontrol through several mechanisms such as the production of antibiotics, iron sequestering compounds, siderophores (Loper and Buyer, 1991; Siddiqui, 2000), extracellular hydrolytic enzymes, other secondary metabolites such as Hydrogen Cyanide (HCN) and induced systemic resistance (Liu et al., 1995). Siddiqui (2000) stated that

"the use of *P. aeruginosa* and *B. subtilis* as seed dressing or as soil drench significantly suppressed root-rot/root-knot infection and nematode population densities under greenhouse and field conditions and thereby enhanced plant growth and yield in mungbean".

Bacillus spp. are known to reduce the wilting index in *F. udum* inoculated plants and improving plant growth (Haseeb et al., 2005).

The present study demonstrates the use of *Bacillus* isolates and *Trichoderma* spp. in an attempt to improve the growth of the infected chickpea plants, reduce wilt/root-rot disease incidence and severity and to increase the yield of chickpea.

MATERIAL AND METHODS

A pot experiment was conducted for three consecutive seasons during winter of 2006/2007, 2007/2008 and 2008/2009, to determine the ability of two isolates of *Bacillus* and two species of *Trichoderma* to reduce disease infection of root lesion nematode (*Pratylenchus* spp.) and *Fusarium* spp.

BACTERIAL INOCULA

Bacillus isolates (B3 and B16) used in this study were isolated from chickpea (*C. arietinum* L.) rhizosphere. These isolates demonstrated the highest *in vitro* antagonism against *F. oxysporum* f.sp. *ciceris* and *F. solani* according to a previous *in vitro* Petri plate bioassay. The bacterial isolates were maintained in tubes containing Nutrient Agar (NA) media in an incubator at 30°C. Culture tubes each containing 10 ml NA media were autoclaved for 30 min at 1 kg/cm^2 pressure. After the culture tubes were cooled, each tube was inoculated at 30°C with the two *Bacillus* isolates (B3 and B16). The culture tubes were then placed in an incubator for 48 hr for the multiplication of the bacterial isolates. For mass production, 500 ml conical flasks containing 300 ml Nutrient Broth was autoclaved at the same pressure and time as before. After the flasks were cooled, each flask was inoculated with a loopful of the cultured bacterial isolates. The flasks were incubated at 28°C on an orbital shaker (Adolf Kuhner AG, Birsfelden Switzerland) at 125 rpm for 48 hr. Inoculum suspension was adjusted by serial diluting in Petri dishes containing NA media, so that the final Colony Forming Unit count (CFU) was maintained at 1×10^8 CFU/ml.

TRICHODERMA SPP. INOCULA

The two species of *Trichoderma* were cultured and maintained in PDA media. *T. harzianum* isolated from the infected chickpea fields at Shambat Research Station and *T. viride* was kindly provided by the Plant Protection Directorate (PPD), Khartoum, Sudan. Active cultures were obtained by sub-culturing in 9 cm Petri dishes containing prepared PDA media supplemented with chloramphenicol (0.05 g/L) as a bacteriostatic agent. The cultures were incubated at 25°C for 7–10 days in darkness. Ten (10) ml of sterilised distilled water were added to each Petri dish, and the surface of the culture was scraped with a glass spreader to dislodge the spores. The spore suspension derived from three Petri dishes from each isolate was transferred separately to a 300 ml sterilised flask. The concentration of the spores was determined with a haemocytometer so that the final fungal count was maintained at 1×10^6 spores/ml.

RAISING AND MAINTENANCE OF TEST PLANTS

Chickpea (*C. arietinum* L.) seeds cv. Shindi were chosen for this study on the basis of their known susceptibility reaction to *F. oxysporum* f. sp. *ciceris*, *F. solani* and *Pratylenchus* spp (Abbo, 2004). Seeds were surface sterilised with 2% sodium hypochlorite for 2 min and rinsed three times in sterilised water. Earthen pots, 20 cm in diameter, were sterilised in an oven at 80°C for 48 hr. Each pot was filled with 2.5 kg of infected soil obtained from the wilt sick plot at Shambat Research Station, Khartoum, Sudan. The control treatment comprises two lots of pots. Five pots were filled with infected soil and the other five pots were filled with autoclaved soil at 80°C for 2 hr. Ten seeds were sown per pot. The pots were kept in a greenhouse. Seeds were placed in the soil surface approximately 2 cm from the top of the pot. Each pot received 10 ml of each of *Bacillus* isolate (B3 and B16) and/or *Trichoderma* spp. (Tv and Th) applied around the chickpea seeds and covered with soil, then immediately irrigated. Control plants received an equivalent amount of sterilised water. The treatments were as follows: B16 alone, B3 alone, Tv alone, Th alone, B16xTv, B16xTs, B3xTv, B3xTh, Control Infected soil (CI) and Control Sterilized soil (CS). Two weeks after planting, seedlings were thinned to three plants per pot, selected for uniformity in growth. Plants were watered daily and fertilised with nitrogen fertiliser once at the rate of 50 Kg/feddan.

DISEASE ASSESSMENT

The disease reaction was assessed by the incidence and severity of Fusarium and Nematode spp. symptoms on individual plants at 7 to 8 days intervals until the end of the experiment. The incidence of foliar symptoms (I), rated 0 or 1, was recorded according to the number of plants infected. The severity of Fusarium wilt was rated on a scale from 0 to 4 (Plate 1), according to the percentage of foliage with yellowing or necrosis (0=0%, 1=1–33%, 2=34–66%, 3=67–100%, 4=death of the plant) adopting the scale proposed by Landa et al. (2001) and Castillo et al. (2003). The incidence of foliar symptoms (I) and severity data (S) was used to calculate a Disease Intensity Index (DII) as follows: DII=(I×S)/4. Thus, DII expresses the mean value of disease intensity at any given moment as a proportion of the maximum possible amount of disease (Castillo et al., 2003). The plant growth parameters are:

1. plant height
2. number of leaves
3. number of branches and
4. number of flowers, were recorded every two weeks after seedling emergence.

All parameters were recorded every two weeks after seedling emergence.

The fresh weight and dry weight were also determined and recorded.

The experiment was carried out in a completely randomised design with five replicates. Duncan's multiple range test was used to test significant differences between treatments at $P \leq 0.05$ (Gomez and Gomez, 1984).

OBSERVATIONS

The plants were uprooted from the pots 105 days after inoculation and the root systems were rinsed gently. Each plant was cut with a knife above the base of the root-emergence zone to separate shoot and root. Shoot and root fresh weights were recorded. Root necrosis was assessed

on a scale of 0–10 according to the percentage of necrotic tissue visible, in which 0=0%, 1=1 to 10% and 10=91 to 100% (Castillo et al., 1998).

The nematode population density in soil and roots was assessed. A 100-g subsample of well-mixed soil from each treatment was processed by the Baermann tray extraction method (Hooper et al., 2005; Southey, 1986). Nematode suspensions were collected in 500 ml flasks after 24 hr, and the number of nematodes counted in five aliquots of 1 ml suspension from each sample was recorded. The means of five counts were used to calculate the population of nematodes per kg of soil. To calculate the nematode number inside the roots, a 1 g subsample of roots was cut into small pieces 1 cm in length, and centrifuged in 10 ml of sterilised water for 4–5 min at 1750 rpm. After spinning, the suspension containing nematode were poured into vials and stored at 4°C for nematode examination and identification, and were then counted under a stereomicroscope (Siddiqui, 2000; Samac and Kindel, 2001). The numbers of nematodes present in the roots were determined per 1 g of root. The average population densities were used to calculate the reproduction index (Rf=final population density divided by initial population density) (Castillo et al., 1995). For dry-weight determination, shoots were kept in envelopes at 80°C in an oven for 48 hr and the weight recorded in grams.

RESULTS

Bacillus isolates and *Trichoderma* spp. used in the experiment has a greater potential as Biocontrol Agents (BCA) for wilt-root/rot disease complex management when incorporated together rather than alone. These BCAs were successful in increasing plant growth parameters and weights, and reducing the infestation and population density of the root lesion nematode *Pratylenchus* spp in soil and roots indicated by the DII.

Inoculation with *Bacillus* isolates and *Trichoderma* spp. and their combinations induced a significant increase ($P \leq 0.05$) in the chickpea growth parameters versus plant height, the number of branches, leaves and flowers compared to the CI plants. Inoculation with *T. harzianum* increased plant height by 53.32% over the other inoculations; however, the greatest increase in number of leaves and flowers was recorded when *Bacillus* isolate B16 was incorporated with *Tv* (47.42% and

Table 1 Effect of *Bacillus* isolates B16 and B3, *Trichoderma viride* (Tv) and *T. harzianum* (Th) and their combinations on the number of the nematode *Pratylenchus* spp. in soil and roots

Treatments	*Percentage number of nematodes (%)*	
	In soil	*In roots*
B16	61.87^{cd}	22.04^{b}
B3	59.56^{c}	18.72^{b}
Tv	60.23^{c}	19.36^{b}
Th	69.79^{cd}	24.36^{b}
B16*Tv	52.05^{bc}	19.64^{b}
B16*Th	45.03^{bc}	19.48^{b}
B3*Tv	40.66^{bc}	19.49^{b}
B3*Th	36.77^{b}	16.32^{b}
CI	100.00^{e}	100.00^{c}
CS	0.00^{a}	0.00^{a}

Note: Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple-range test.

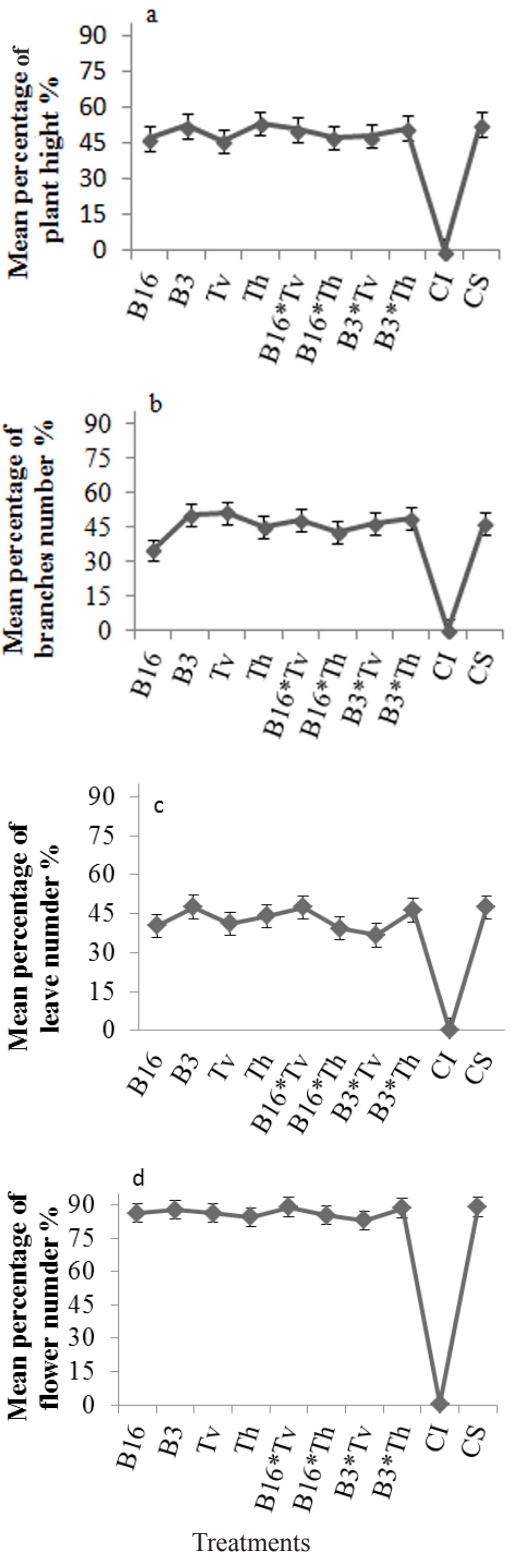

Figure 1 The percentage effect of *Bacillus* isolate B16 and B3, *Trichoderma viride* (Tv) and *T. harzianum* (Th) with their combinations on (a) plant height, (b) number of branches, (c) number of leaves and (d) number of flowers per plant, compared to CS and infected plants (CI). Error bares represent standard error for means. Each point is the mean of five replicates for three seasons (2006/07–2007/08–2008/09)

88.83%, respectively). Inoculating with *T.viride* alone increased the number of branches by 51.32% (shown in Figure 1).

On average, plant inoculation with *Th* produced a pronounced reduction effect on almost all the tested parameters affecting nematode progress. It reduced the number of nematodes in soil and roots (30.21% and 75.63%, respectively) with a DII of 0.52 compared to 3.29 in CI plants (Table 1). When *Bacillus* isolate B3 was combined with *T. harzianum,* it reduced the severity of root necrosis by 2.74 and the reproductive factor by 1.19 compared with CI plants (8.16 and 5.94, respectively) (Table 2). No significant reduction levels were recorded between all the tested antagonisms in the DII. However, the applied combination of *Bacillus* isolate B16 and *T.viride* gave the least reduction level of 0.44 (Table 2).

Chickpea fresh root and dry shoot weights were highly affected when inoculated with the mixed application, with no significant difference between applications. The application of *T. harzianum* and *Bacillus* isolate B16 was the most effective application, recording a high increase in fresh root and dry shoot weight percentages of 58.22% and 53.60%, respectively (Figure 2).

The effect of the bacterial and fungal antagonisms on the disease progress of Fusarium wilt on chickpea plants, as illustrated in Figure 3, demonstrated that all the applied antagonisms have the ability to decrease the progress of the Fusarium wilt disease from day 15 after inoculation forward. However, the combined applications were more highly effective in reducing the disease progress curve than the individual ones, especially *Bacillus* isolate B16 when combined with the two *Trichoderma* spp. (Figure 3).

DISCUSSION

In the current study, chickpea plants (*Cicer arietinum* L.), variety Shindi, were found highly susceptible to *Fusarium* wilt/root-rot disease complex, as indicated by the high records of root necrotic severity, nematode population densities and plant growth suppression in comparison

Table 2 Effect of *Bacillus* isolates B16 and B3, *Trichoderma viride* (Tv) and *T. harzianum* (Th) and their combinations on the severity of root necrosis, the reproduction index (Rf) and the disease intensity in chickpea plants

Treatments	*Severity of Root necrosis*[†]	*Rf %*[α]	*DII*[μ]
B16	3.88^{bcd}	53.68^{c}	0.56^{b}
B3	4.44^{d}	55.18^{cd}	0.55^{b}
Tv	4.31^{d}	53.30^{c}	0.52^{b}
Th	4.06^{bcd}	60.78^{d}	0.45^{b}
B16*Tv	3.33^{bcd}	43.88^{bc}	0.44^{b}
B16*Th	3.20^{bcd}	34.97^{ab}	0.54^{b}
B3*Tv	4.22^{cd}	29.00^{ab}	0.52^{b}
B3*Th	2.74^{b}	22.09^{a}	0.45^{b}
CI	8.16^{e}	100.00^{e}	3.29^{c}
CS	0.00^{a}	0.38^{a}	0.00^{a}

[†]Severity of root necrosis was assessed on a scale of 0–10 according to the following scale, 0=0%, 1=1 to 10% and 10= 91 to 100%.

[α]Rf (The reproduction index)=final population density divided by initial population density.

[μ]DII (The disease intensity index)=incidence of foliar symptoms×severity data/4, after 90 days of inoculation.

Note: Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Duncan's multiple-range test.

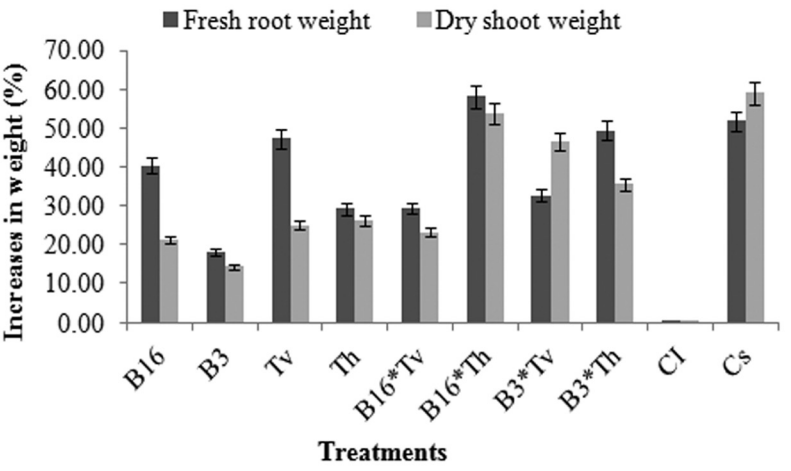

Figure 2 The percentage effect of *Bacillus* isolates (B16 and B3), *Trichoderma viride* (Tv) and *Trichoderma harzianum* (Th) and their combinations on fresh root weight and dry shoot weight on chickpea susceptible plants. Each column is the mean of 5 replicates for three seasons (2006/07–2007/08–2008/09). Vertical bars represent standard errors

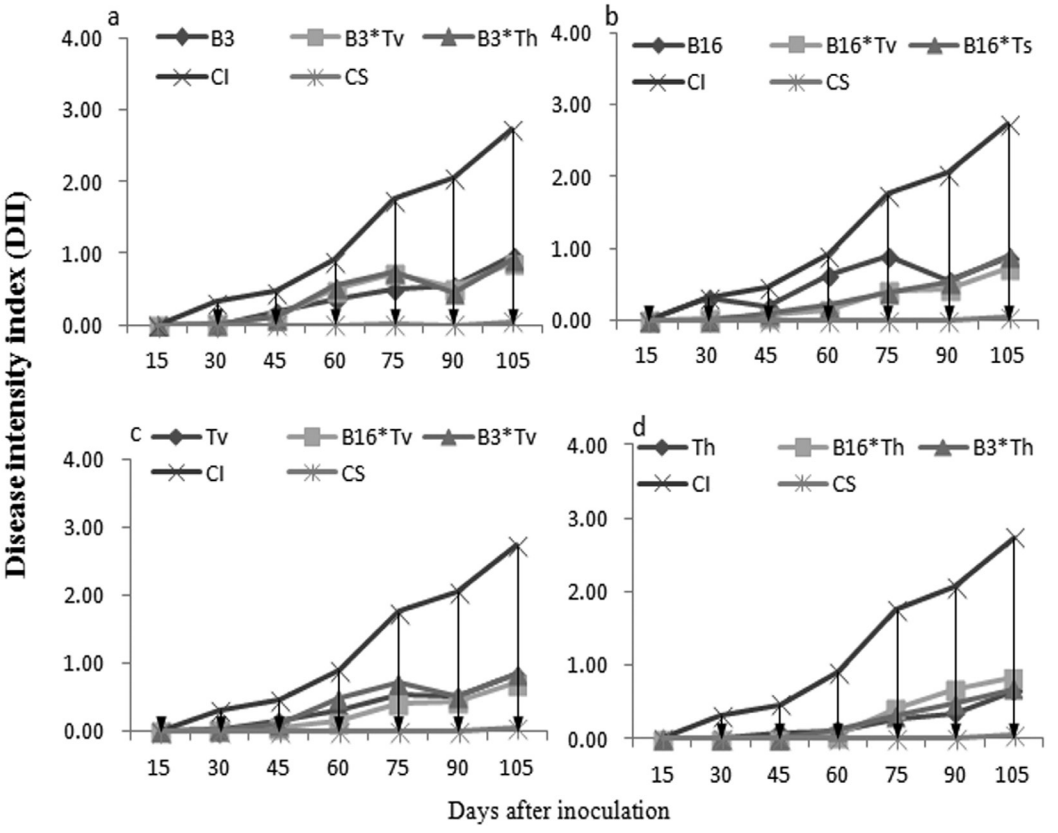

Figure 3 Disease progress of Fusarium wilt in chickpea plants grown in infected soil and treated with *Bacillus* isolate B16 (a), *Bacillus* isolate B3 (b), *T.viride* (c) and *T.harzianum* (d) and their combinations compared to CS and infected plants (CI). Each drop lines represent the progress of the disease compared to control (CI). Each point is the mean of DII of five replicates for three seasons (2006/07–2007/08–2008/09); each replicate consists of three potted plants

with the controls. The present results indicated that the combination of the BCA was more effective than either used alone in reducing disease progress and/or promoting plant growth. As reported by Duffy et al. (1996), Larkin and Fravel (1998), Howell (2003) and Maketon et al. (2008), the use of a combination of multiple antagonistic organisms may provide improved disease control over the use of a single organism. They enhance the level and consistency of control by providing multiple mechanisms of action, more stable rhizosphere community, and are effective over a wider range of environmental conditions. In particular, combinations of fungi and bacteria may provide protection at different times or under various conditions, and occupy different or complementary niches. Such combinations may overcome inconsistencies in the performance of individual isolates. A single biocontrol agent may not perform well at all times in all kinds of soil environment to suppress plant pathogens (Raupach and Kloepper, 1998).

The mixed inoculation of *Bacillus* isolates and *Trichoderma* spp. enhanced plant growth expressed by an increase in plant height, number of branches, leaves, flowers and fresh and dry matter accumulation and the suppression of the disease complex up to 100% compared to controls. This is represented by the severity of root necrosis and DII, better than individual applications and in a shorter time period. The promotion of chickpea growth parameters by *Bacillus* isolates and *Trichoderma* spp. may be due to their ability to produce phytohormones, vitamins and solubilising minerals in addition to their role in the direct inhibition of pathogen growth (Morsy et al., 2009).

The individual effect of the *Bacillus* isolates in promoting plant growth was rarely observed (Figure 1). The combined effect of *T. harzianum* with either of the *Bacillus* isolates was more effective than the inoculation with *Tv*, which was evidenced in the enhanced growth in most of the parameters and disease reduction percentages. These results are in agreement with Abeysinghe's (2007) findings that observed that protection of bean seedlings was more pronounced in *T. harzianum* RU01 treated plants than bacterized plants with *B. subtilis* CA32, and enhanced root growth was observed only in *T. harzianum* RU01 treated plants. This suggests that biotic modification of the mycorrhizosphere was a result of colonisation with *T. harzianum* RU01.

The findings of Maketon et al. (2008) showed that neither *B. subtilis* AP-01 nor *T. harzianum* AP-001 alone could control the bacterial wilt, damping-off and frogeye leaf spot of tobacco diseases, but, when combined, their controlling capabilities increased. Monte (2001) explained this finding as a result of an augmentation of levels of inhibitory compounds, which was not seen when either antagonism was applied alone. Hence *Trichoderma* strains strongly stimulate plants to produce their own antimicrobial compounds.

The *Trichoderma* spp. survive in the soil by using organic matter as their primary source of nutrients and nematodes as a secondary source. Thus, their behaviour in the soil ecosystem to some extent mirrors that of predacious fungi that use nematodes as a food base when under nutritional stress (Sikora et al., 2005). The fungi's lack of dependence on nematodes for growth and survival in soil is reflected by the fact that the DII is not correlated with nematode density (Tables 1 and 2 and Figure 3). This finding was recorded when chickpea plants were treated with *Trichoderma* spp. In this study, the inoculation of *Trichoderma* spp. singly was less effective in nematode control compared to the mixed applications. Organic matter composition and the associated biotic and abiotic factors can affect the activities of Trichoderma, especially in relation to the conduciveness/receptivity of the soil to the strain (Vinale et al., 2008).

The mechanisms of action used by Trichoderma, competition, antibiosis, parasitism and systemic-induced resistance, are influenced by concentration and availability of nutrients (carbohydrates in lignocellulosic substances, chitin, lipids, etc.) within the soil's organic matter (Hoitink et al., 2006).

Moreover, a high percentage of nematode population density in soil was recorded even though a minimum percentage of the average of nematode population density in soil was estimated (Table 1). These were higher than Castillo et al.'s (1998) estimation (1000 nematode/100 g) in infected chickpea plants with lesion nematodes, irrespective of fungal inoculum or nematode densities.

The high percentage of nematode population density within soils (36.77–69.79%) compared to that in roots (16.32–24.36%) (Table 1) may be attributed to the induced resistance capacity of both antagonists (Castillo et al., 1998; Hoitink et al., 2006) and to the explanation of Monte (2001), although the number of nematodes in roots was lower compared to chickpea susceptible lines and cultivars used in Castillo experiments (Castillo et al., 1995). According to an experiment conducted by Siddiqui et al. (2001), they stated that

> "despite reduction, final populations remained at fairly high levels, presumably because short-duration colonization results in delayed nematode hatch and ultimate penetration".

In addition, only a portion of the roots and root tips are colonised with the bacterial and fungal antagonisms, and therefore a certain amount of root penetration always occurs. The combined inoculation of *T. harzianum* with *Bacillus* isolate B16 resulted in a great increase in fresh root and dry shoot weight, and reduced the population density of *Pratylenchus* spp. in soil and roots by 73.38% and 86.94%, respectively, compared to that reported by Bhatti and Kraft (1992), who stated that a "simultaneous application of *P. fluorescens* plus *T.harzianum* and *T.viride* separately decreased *P. thornei* population in soil and root by 73% and 61%, respectively".

All the treatments were effective in reducing the disease, indicated by the reduced severity of root necrosis, which was in the range of 2.71–4.44 in treated plants, whereas the range in the infected soil reached 8.3. Even the nematode reproductive index, indicated by Rf, was highly reduced by the co-application of *Bacillus* isolate B3 with *Th*, with a reduction range of 22.09%. This is more effective than *P. fluorescens* plus *T.viride* when applied to control *P. thornei* in chickpea plants, and resulted in a final nematode population reduction of 22.30% (Dwivedi and Johri, 2003). Most rhizobacteria act against plant-parasitic nematodes by means of metabolic by-products, enzymes and toxins. The effects of those toxins include the suppression of nematode reproduction, egg hatching and juvenile survival, as well as direct killing of nematodes (Tian et al., 2007). Moreover, Morsy et al. (2009) reported that *B. subtilis* can secrete several antifungal metabolites such as subtilin, bacitracin, bacillin and bacillomycin, which have an inhibitory effect on fungal pathogens.

The fungal and bacterial antagonists survive in the soil by using organic matter as their primary source of nutrients and nematodes as a secondary source, and use nematodes as a food base when under nutritional stress. Their lack of dependence on nematodes for growth and survival in soil is reflected by the fact that the severity of root necrosis is not correlated with nematode density (Sikora et al., 2005).

CONCLUSIONS

According to the findings of this study, the following conclusions can be drawn:

- The use of the selected plant growth promoting rhizobacteria *Bacillus* isolates and the fungal antagonism *Trichoderma* spp. are antagonistic to both fungi and nematodes.
- They could be developed into a valuable crop management programme to reduce the deleterious impact of soil-borne pathogenic fungi and plant parasitic nematodes on plant growth.

Successful use of microbial antagonists will depend upon first obtaining a thorough understanding of their ecological behaviour, followed by studies of their physiology and genetics.

Future research studies should concentrate on the metabolites the microbes produce and what happens when they are inoculated in combination, since individual inoculation of these microbes gave less control of plant diseases.

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