MANAGEMENT OF *MELOIDOGYNE INCOGNITA* AND *FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI* WILT COMPLEX USING ANTAGONISTIC FUNGI IN TOMATO

M. Nagesh*, S.S. Hussaini, B. Ramanujam and B.S. Chidanandaswamy

Project Directorate of Biological Control, Post Box No. 2491,
H. A. Farm P. O., Bellary Road, Bangalore 560 024, India

Summary. Two beneficial fungi *viz*., *Paecilomyces lilacinus* antagonistic to phytoparasitic nematodes and *Trichoderma harzianum* antagonistic to pathogenic fungi, in combination with neem cake, were evaluated for the control of the root-knot nematode *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *lycopersici* wilt complex. The three fungi were individually examined for variations in their growth and sporulation and their mutual interactive effects (dual culture method) on potato dextrose agar medium at 28 °C. The interrelationship between *Meloidogyne incognita* and *F. oxysporum* f. sp. *lycopersici* in susceptible tomato cv. Pusa Ruby, was studied and their management by the integrated approach of using *P. lilacinus* and *T. harzianum* and neem cake was evaluated in pots in a glass-house. Among the three fungi, *T. harzianum* PDBC TH23 grew and sporulated faster (8.8 cm in 7 days and sporulation after 7 days) than *P. lilacinus* PDBC PL55 (6.8 cm in 7 days and 14 days, respectively) and *F. oxysporum* (4.9 cm in 7 days and 18 days, respectively). In dual culture studies, the combinations of *P. lilacinus* with *T. harzianum* or *F. oxysporum* did not exhibit any mutual inhibition/suppression, while the combination of *T. harzianum* + *F. oxysporum*, resulted in 42% inhibition of *F. oxysporum*. Inoculation of *M. incognita* juveniles prior to *Fusarium* inoculation advanced the wilt of tomato by a week, while nematode inoculation after *Fusarium* inoculation did not increase wilting. *Trichoderma harzianum* controlled wilt in the presence of *M. incognita* for 6 weeks after transplanting. Roots of tomatoes that received *P. lilacinus* along with *T. harzianum* and neem cake were free from nematode and did not wilt till harvest. The roots obtained from these combinations were free from *Fusarium* fungus.

Key words: Biological control, neem cake, *Paecilomyces lilacinus*, root-knot nematode, *Trichoderma harzianum*.

Disease complexes involving nematodes and fungal pathogens may cause significantly more crop losses than individually (Hussey and McGuire, 1987). Synergistic interactions between nematodes and fungi have been recognized since 1892, when Atkinson (1892) reported that infection of cotton by root-knot nematodes increased the severity of *Fusarium* wilt. Such interactions are considered synergistic when the combined effects of two pathogens on the host plant result in more extensive damage than the sum of the effects when both are acting independently (Powell, 1979; Wallace, 1983). Powell (1979) emphasized that although the synergistic complexes may be common in crop production, only a few nematode-disease complexes have been described.

Disease management strategies have evolved to specifically mitigate the damage caused by the combined effects of nematode-fungal pathogen interactions. Soil fumigation with a nematicide reduced population densities of *Meloidogyne incognita* (Kofoid *et* White) Chitw. by 70% and *Fusarium* wilt incidence by 81%, increasing yield by 77% in a cotton cultivar susceptible to root-knot-*Fusarium* wilt complex (Shepherd, 1982). Several wilt resistant/tolerant cultivars or accessions of crop plants not only exhibit severe wilting but also advance the expression of wilt in the presence of plant parasitic nematodes (Garber *et al.*, 1979; Nagesh *et al.,* 1998). Mai and Abawi (1987) found that the control of root-knot nematodes resulted also in a great reduction of the incidence and severity of *Fusarium* wilt. Chemical control (fumigation) in soils with combined infestation of *Fusarium* wilt and root-knot nematode is expensive and temporary, while crop breeding for combined resistance/tolerance to nematode-fungal wilt complex is a long-term approach. Improvement of soil antagonistic potential by biological control offers a cost-effective, eco-friendly, target-specific and sustainable alternative for the management of nematode-disease complexes. The antagonistic potential of *Trichoderma* sp. and *Gliocladium* sp. against several soil-borne fungal pathogens were well documented and led to their commercial use (Papavizas, 1985). Cliquet and Scheffer (1996) demonstrated the success of biological control of damping-off caused by *Pythium ultimum* Trow and *Rhizoctonia solani* Kuhn using *Trichoderma* spp. Agbenin *et al.* (2004) observed that treatment with 2 g/kg soil of neem seed powder in the screenhouse and 2000 kg/ha in the field significantly reduced the disease severity of *Fusarium* and root-knot nematode in tomato cultivar Roma VF

* Corresponding author: Senior Scientist, Project Directorate of Biological Control, Post Box No. 2491, H. A. Farm P.O., Bellary Road, Bangalore 560 024, India. nagesh55@yahoo.com; pdblc@kar.nic.in

in both screenhouse and field. However, experimental evidence regarding the possibility of managing *Fusarium*/root-knot- nematode wilt complex in tomato using a combination of two antagonists simultaneously and neem cake is lacking. Therefore, pot experiments were conducted to study the effect of integration of two antagonistic fungi viz., *Paecilomyces lilacinus* (Thom.) Sampson (an egg parasite of nematodes) and *Trichoderma harzianum* Rifai (an antagonist of *Fusarium* wilt), with neem cake on *Meloidogyne incognita*, *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder *et* Hansen and on the host plant tomato. Further, *in vitro* inter-relationship studies were carried out using these fungi in order to observe their growth as dual cultures.

MATERIALS AND METHODS

Pure cultures of *Paecilomyces lilacinus* isolate PDBC PL55, initially isolated from root-knot nematode infested tomato fields located in Kolar, Karnataka, India (Anonymous, 2002) and *Trichoderma harzianum* isolate PDBC TH23 (isolated from a suppressive soil located in Devanahalli, Bangalore, India) were obtained by single spore isolation, maintained on potato dextrose agar (PDA) slants at 28 ± 1 °C and multiplied on autoclaved wheat bran at 28 ± 1 °C. Similarly, a local isolate of the root pathogen, *Fusarium oxysporum* f. sp. *lycopersici* (Indian Institute of Horticultural Research, Bangalore, India), isolated from field samples of tomato roots, was multiplied on PDA at 28 ± 1 °C (Nash and Snyder, 1962).

Maintenance of the fungal isolates. One set of these isolates were inoculated to 0.5 ml of sterile potato dextrose broth (PDB) in 1.5 ml Eppendorf tubes separately, incubated at 28 °C for seven days and 0.5 ml of autoclaved glycerol was added in each tube. These tubes were maintained at -20 °C as stocks. Another set of pure cultures of the isolates were maintained in Petri plates at 4 °C and used for further experiments.

In vitro *studies on inter-relationships of fungi.* The growth rate and sporulation time of the isolates of *F. oxysporum* f. sp. *lycopersici, P. lilacinus* and *T. harzianum* under study were examined individually by placing 9-mm-diameter mycelial discs of each fungus in the centre of the Petri-plates containing sterile potato dextrose agar medium (PDA) and incubated at 28 ± 1 °C for ten days. Radial growth in diameter and time of apparent completion of sporulation by each fungus in each Petri-plate were recorded. The values of these cultures were considered as controls for comparison.

The reciprocal effects between these fungi were studied by dual culture technique (Dennis and Webster, 1971). In one set, 9-mm-diameter mycelial discs of *F. oxysporum* f. sp. *lycopersici* were placed at one side of the 90-mm-diameter Petri-plates with PDA followed by 9-mm-diameter discs of *P. lilacinus* at the opposite side. In a second set, 9-mm-diameter discs of *F. oxysporum* f. sp. *lycopersici* and *T. harzianum* were placed and, in a third set, 9-mm-diameter discs of *P. lilacinus* and *T. harzianum* were placed. Five replications were prepared for each set and all were incubated at 28 ± 1 °C for ten days. Subsequently, the radial growth of the colonies was measured and the growth inhibition was calculated as (A-B)/A × 100, where A = Control measurement, B = averages of replicates.

Nematode culture. Root-knot nematode second stage juveniles (from single egg masses) were continually cultured on eggplant in an earthen pot containing autoclaved sandy loam soil. Healthy egg masses were collected individually from the roots of eggplant, cleared of soil, washed in distilled water and then kept on Baermann funnels at 30 °C to stimulate egg hatch. Freshly emerging juveniles (collected daily for six days and stored at 8 °C in distilled water) were used for the experiments.

Pot experiments. The effect of integration of two antagonistic fungi viz., *P. lilacinus* (PL) and *T. harzianum* (TH), on the nematode-wilt disease complex and on tomato (*Lycopersicon esculentum* Mill.) was studied in a glasshouse. Earthen pots of 30-cm diameter were filled with autoclaved soil (2500 cm^3 of red loamy soil with pH 6.3 and organic carbon 2.35%). Thirty-day-old healthy tomato cv. Pusa Ruby seedlings were planted (two per pot) and established in pots designated as treated and untreated (autoclaved check). The pots designated for *M. incognita* and *F. oxysporum* treatments were inoculated with the nematode and the fungus and mixed thoroughly. Treatments were: *Meloidogyne incognita* (MI) (2,000 J_2/pot); *Fusarium oxysporum* f. sp. *lycopersici* (FO); MI + FO; FO + MI; MI + FO + PL; MI + FO + TH; MI + FO + Neem cake; MI + FO + PL + NC; MI + FO + TH + NC; MI + FO + PL + TH + NC and uninoculated autoclaved check. The application rates of the fungi were: *F. oxysporum* 4 g PDA laden with 1.2×10^8 spores/g; *T. harzianum* PDBC TH23 5 g PDA laden with 2.2×10^8 spores/g); *P. lilacinus* PDBC PL55 5 g PDA laden with 1.2×10^8 spores/g. Neem cake was added at the rate of 20 g/plant. In all the treatments, the nematode juveniles + *Fusarium* were inoculated first, followed by neem cake, *T. harzianum* and or *P. lilacinus* after 7 days, in order to establish the pathogens first and make the soil MI + *Fusarium* sick, so simulating the natural situation of nematode-disease complex. The sequence of inoculation between the nematode and *Fusarium* was: in the case of FO + MI treatment, tomato plants were inoculated first with *Fusarium* followed by juveniles of MI after 7 days, while in MI + FO treatment, tomato plants were inoculated first with MI followed by FO after 7 days. After the incorporation of all the treatments into the soil, the pots were irrigated and left for five days to allow the fungi to establish. Ten replicates were prepared for each treatment.

Wilt symptoms were recorded at weekly intervals

starting on the tenth day after transplanting and for the following eight weeks. Wilting was rated according to the scale given in Table III.

Observations on healthy and *Fusarium* infested plants, number of plants that recovered, root colonization by antagonistic fungi and colonization of egg masses by the fungi were recorded. Root galling was rated according to the scale suggested by Taylor and Sasser (1978). The second stage juveniles of the nematode at the end of the experiment (Pf), were extracted from 100 cm^3 soil by the Baermann's funnel method and the reproduction rate of the nematode (Pf/Pi) was calculated by dividing Pf with the number of nematode juveniles inoculated (Pi). To determine the root colonization by each antagonistic fungus, the roots of tomatoes collected from each treatment were washed thoroughly, cut into approximately 1-cm-long pieces, surface sterilized with 0.1% NaOCl for 45 seconds and placed in Petri plates containing semi-selective media for each fungus separately. The media defined by Mitchell *et al.* (1987) was used for *P. lilacinus* and that defined by Elad and Chet (1983) was employed for *T. harzianum*. Root colonization by *P. lilacinus* and *T. harzianum* in plants that received their combined applications were recorded separately on their respective selective media and added together to express as total per cent root colonization. Similarly, 50 egg masses from each treated root system were collected separately, washed in sterile water, surface sterilized in 0.01% NaOCl for 20 seconds and transferred into Petri plates containing semi-selective medium for *P. lilacinus* or *T. harzianum*. The cultures were incubated at 30 °C for five days and the fungal development and inhibition were recorded every day. Another set of 50 egg masses was collected and placed in 0.01% NaOCl for 45-50 seconds (until the egg masses dissolved and released eggs freely into the suspension), washed 5 times in sterile distilled water (Hussey and Barker, 1973) and concentrated using a table-top microcentrifuge at 500 rpm. The final egg suspension was diluted to give 1000 juveniles per ml. Aliquots of 100 µl containing 100 surface-sterilized eggs of *M. incognita* were added to the Petri-plates containing a thin layer (~1mm) of *P. lilacinus* and *T. harzianum* semi-selective PDA media and incubated at 28 ºC. The eggs were observed under a stereo-zoom-microscope (at 40×) at 24-hour interval for seven days to record fungal growth and infection. Observations were recorded in four replicated plates and the mean values were estimated.

All data were subjected to analysis of variance and the means compared by Duncan's multiple-range test.

RESULTS

In vitro *studies on inter-relationships of fungi.* Among the three fungi under study, *T. harzianum* PDBC TH23 showed the most rapid mycelial growth followed by *P. lilacinus* PDBC PL55 and *F. oxysporum* f. sp. *lycopersici* on PDA at 28 °C (Table I). Similarly, *T. harzianum* completed sporulation on PDA at 28 °C in 7 ± 1days, while *P. lilacinus* and *F. oxysporum* f. sp. *lycopersici* required 14 ± 1 and 18 ± 1 days, respectively (Table I). The daily growth rate of *T. harzianum* was 1.12 cm, which was the greatest among the three fungi, followed by that of *P. lilacinus* (0.84 cm) and *F. oxysporum* f. sp. *lycopersici* (0.57 cm).

In dual culture studies on fungi interaction, only a discernible inhibition in mycelial growth was observed for *F. oxysporum* f. sp. *lycopersici* (42.5%) in the presence of *T. harzianum* (Table II). Growth inhibitions were of 5% for *F. oxysporum* f. sp. *lycopersici* in the presence of *P. lilacinus* and of 7.1% and 2.3% for *P. lilacinus* in the presence of *T. harzianum* and *F. oxysporum* f. sp. *lycopersici*, respectively. Similarly, there was a marginal reduction in the mycelial growth of *T. harzianum* (6.6% and 2%, respectively) in the presence of *P. lilacinus* and *F. oxysporum* f. sp. *lycopersici*. *In vitro* studies showed that *T. harzianum* not only grew faster but inhibited the growth of *F. oxysporum* f. sp. *lycopersici* by 42.5%. Further, *P. lilacinus* did not inhibit the growth of *F. oxysporum* f. sp. *lycopersici* or *T. harzianum*, indicating that it had no antagonistic effect on either fungus. Since the growth rate of *P. lilacinus* was slower than that of *T. harzianum,* application of *P. lilacinus* would have to be earlier for better establishment and to avoid competition in soil i.e., in the host plant rhizosphere.

Table I. Growth and sporulation of *Trichoderma harzianum, Paecilomyces lilacinus* and *Fusarium oxysporum* f. sp. *lycopersici* on potato dextrose agar medium (PDA) at 28 ºC.

Fungus	Mycelial growth on PDA at 28 ºC (cm)		Sporulation on PDA at 28 ºC	
	2days	7days	Initiation (Days)	Apparent completion (Days)
T. harzianum PDBC TH23	5.2±0.4 (4.3)* (2.1)**	8.8±0.2 (7.9)* (1.12)**	4±1	7±1
P. lilacinus PDBC PL55	1.6±0.2 (0.7)* (0.35)**	6.8±0.3 (5.9)* (0.84)**	9±1	14±1
F. oxysporum lycopersici	1.2±0.2 (0.3)* (0.15)**	4.9±3 (4.0)* (0.57)**	13±1	18±1

Values in parentheses indicate: *Increase in mycelial growth in diameter for 2 days; **Increase in mycelial growth in diameter per day over 0.9-cm-diameter of mycelial discs.

Table II. Interactive effect of *T. harzianum, P. lilacinus* and *F. oxysporum* f. sp. *lycopersici* under dual culture technique on potato dextrose agar medium (PDA) at 28 °C.

Fungus	*F. oxysporum* f. sp. *lycopersici*		*P. lilacinus* PDBCPL55		*T. harzianum* PDBCTH23	
	Radial growth (7 days)	Per cent inhibition over control	Radial growth (7 days)	Per cent inhibition over control	Radial growth (7 days)	Per cent inhibition over control
P. lilacinus PDBC PL55	3.8±0.3	5.0	4.2±0.2*	-	4.5±0.2	6.6
T. harzianum PDBC TH23	2.3±0.2	42.5	3.9±0.2	7.1	4.8±0.3*	-
F. oxysporum lycopersici	4.0±0.2*	-	4.1±0.3	2.3	4.7±0.3	2.0

*Respective Controls.

Table III. Progress of *Fusarium* wilt in tomato in the presence of *Meloidogyne incognita,* bioagents and neem cake combinations.

Treatment	Wilt index* at weekly intervals starting 10 days after transplanting							
	1	2	3	4	5	6	7	8
M. incognita (MI)	0	0	0	0	0	0	0	0
F. oxysporum lycopersici (FO)	0	0	+	++	++	-	-	-
MI + FO (MI inoculated 7 days prior to FO)	0	+	+	++	++	-	-	-
FO + MI (FO inoculated 7 days prior to MI)	0	0	+	++	++	-	-	-
MI + FO + *P. lilacinus* PDBC PL55 (PL)	0	0	0	+	+	++	++	-
MI + FO + *T. harzianum* PDBC TH23 (TH)	0	0	0	+	+	+	++	-
MI + FO + Neem cake (NC)	0	+	+	++	++	-	-	-
MI + FO + PL + NC	0	0	0	0	0	+	++	++
MI + FO + TH + NC	0	0	0	0	+	+	+	0
MI + FO + PL + TH + NC	0	0	0	0	0	0	+	0

Wilt Index*: 0 = No wilting; + = wilting of young leaves; ++ = wilting of whole plant; - = Dead plant.
Treatments: MI = *Meloidogyne incognita*; FO = *F. oxysporum lycopersici*; PL = *P. lilacinus* PDBC PL55; TH = *T. harzianum* PDBC TH23; NC = Neem cake. In MI + FO combinations, MI was inoculated 7 days prior to FO; in FO + MI, FO was inoculated 7 days prior to MI. NC, PL and or TH were applied 7 days later.

Pot experiments. There were no wilt symptoms in tomato inoculated with *M. incognita* alone, while tomatoes inoculated with a combination of *M. incognita* and *F. oxysporum* f. sp. *lycopersici* exhibited wilting from the third to the fifth week and subsequently died (sixth week of observation) (Table III). Inoculation of nematodes prior to *Fusarium* inoculation advanced the wilt in MI + FO and MI + FO + neem cake treated tomatoes by a week, while nematode inoculation after *Fusarium* inoculation resulted in wilt incidence similar to that of *Fusarium* inoculation alone.

Plants that received *M. incognita* + *F. oxysporum* f. sp. *lycopersici* + P. *lilacinus,* and *M. incognita* + *F. oxysporum* f. sp. *lycopersici* + *T. harzianum*, exhibited wilting after seven weeks and subsequently died, indicating that *P. lilacinus* and *T. harzianum* individually controlled *Fusarium* wilt in the presence of *M. incognita* for seven weeks only. Neem cake, when applied singly to plants that received MI + FO inoculations, showed wilt symptoms similar to that of MI + FO, which indicated that it did not suppress or promote tomato wilt in the nematode-wilt complex. Integrated use of *P. lilacinus* + *T.*

harzianum + neem cake on nematode inoculated plants controlled wilt, with 80% of tomato plants free from wilt and 20% that exhibited wilt initially but recovered thereafter.

The greatest numbers of healthy and live plants were found in *P. lilacinus* + *T. harzianum* + neem cake treatment followed by that of *T. harzianum* + neem cake and *P. lilacinus* + neem cake (Table IV). Root-knot index was significantly less in P.L. + T.H. + neem cake and P.L.+ neem cake treated plants than in the other treatments. Nematode reproduction rate was less than one in all the treatments. Tomato root colonization by the two antagonistic fungi was greatest in P.L. + T.H. + neem cake treated plants followed by that in T.H. + neem cake treated plants and P.L. + neem cake treated plants. Comparatively greater colonization of egg masses and parasitization of eggs were recorded in plants that received *P. lilacinus* treatment, including the fungus alone, than in those that received P.L. + neem cake or P.L. + T.H. + neem cake (Table IV).

DISCUSSION

Among the three fungal isolates examined individually in Petri plates for growth and sporulation, *T. harzianum* PDBC TH23 was found to grow and sporulate faster than the other two fungi. The results of dual culture studies established that *P. lilacinus* PDBC PL55 and *T. harzianum* PDBC TH23 did not exhibit mutual inhibition or suppression, whereas *T. harzianum* PDBC TH23 exhibited significant growth inhibition of *F. oxysporum* f. sp. *lycopersici*. This confirmed that *T. harzianum* (isolate PDBC TH23) was antagonistic to *F. oxysporum* f. sp. *lycopersici.* Also, *Paecilomyces lilacinus* and *F. oxysporum* f. sp. *lycopersici* did not record any mutual inhibition. The significance of the findings of interactive studies are: *i*) the mutual compatibility and antagonism of the beneficial and pathogenic fungi, which helps in deciding the compatible combinations of antagonists; *ii*) absolute and differential growth rates of the fungi under study in order to coordinate the application of each fungus to avoid competition for space and nutrition and better establishment in a complex soil environment, which helps in deciding the time and dose of application. These observations suggest that these two antagonistic fungi could be used together to control nematode-wilt complex.

Pot trials in the greenhouse demonstrated that *i*) the isolate of *Fusarium* was virulent to tomato and *ii*) *M. incognita* is an important predisposing factor for the onset and severity of *Fusarium* wilt in tomato. Earlier, Sidhu and Webster (1977) reported that *M. incognita* pre-

Table IV. Effects of integrated use of *P. lilacinus*, *T. harzianum* and neem cake on tomato, *M. incognita* and *Fusarium oxysporum*.

Treatment	Live and healthy plants (%)	Nematode multiplication rate (Pf/Pi in 100 cc soil)	Root-knot index (0-5 scale)	Colonization/parasitization by (*T. harzianum*/*P. lilacinus*/combined)		
				Root colonization (%) (50 root bits examined)	Parasitized eggs/colonized egg masses[1]	
					Egg masses (%)	Eggs (%)
MI + FO	10 a	0.72	4.4 c	-	-	-
MI + FO + PL	30 b	0.58	3.6 b	48 a	48 b	46 b
MI + FO + TH	40 c	0.64	4.0 b,c	50 a	44 b	32 a
MI + FO + NC	10 a	0.66	4.2 b,c	-	-	-
MI + FO + PL + NC	60 d	0.70	2.8 a,b	62 b	62 d	58 c
MI + FO + TH + NC	70 e	0.72	3.8 b,c	68 c	36 a	32 a
MI + FO + PL+ TH + NC	90 f	0.78	1.8 a	74 d	56 c	44 b
F-Test	*	*	*	*	*	*
SEM ±	2.88	0.22	0.66	1.18	1.12	0.99
CD (P = 0.05)	8.87	0.74	1.78	3.66	4.11	3.22

Values are means of five replicated plates.
[1]50 egg masses and 50 eggs/egg masses examined.
*Significant at P = 0.05.
Means followed by the same letter within a column are not significantly different according to modified Duncan's multiple range test.
Treatments: MI = *Meloidogyne incognita*; FO = *F. oxysporum lycopersici*; PL = *P. lilacinus* PDBC PL55; TH = *T. harzianum* PDBC TH23; NC = Neem cake. In MI + FO combinations, MI was inoculated 7 days prior to FO; in FO + MI, FO was inoculated 7 days prior to MI. NC, PL and or TH were applied 7 days later.

disposed tomato to *F. oxysporum lycopersici.* Treatment of tomato plants with *P. lilacinus, T. harzianum,* neem cake or their combinations inhibited the onset of wilt until at least 4 weeks as observed in the case of MI + FO + PL and MI + FO + TH. The approach/strategy of integrating two antagonistic fungi (*P. lilacinus* for the control of root-knot nematode and *T. harzianum* for the control of *F. oxysporum* f. sp. *lycopersici*) with neem cake (organic substrate for the inoculants) effectively delayed the onset and reduced the damage due to *Fusarium*-nematode wilt complex. Chaitali *et al.* (2003) observed that *Trichoderma viride* combined with neem cake controlled *M. incognita* and the root-rot fungus *Rhizoctonia bataticola* Taub Butler (*Macrophomina phaseolina* Ashby) disease complex better than *T. viride* combined with groundnut cake in okra. Also, Nagesh *et al*. (1998) observed that planting of gladiolus corms pretreated with combinations of *T. harzianum, T. viride, P. lilacinus* and neem cake resulted in wilt-free gladiolus, normal flowering and Fusarium-free subsequent corms and cormels.

Application of *P. lilacinus* at least 7-10 days prior to the application of *T. harzianum* is expected to favour its establishment. Finally, the pot experiments indicated the possibility of using two fungal antagonists to control two different pathogens in fields and polyhouses.

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