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RESEARCH ARTICLE

Management of *Fusarium oxysporum* f. sp. *lycopersici* and root-knot nematode disease complex in tomato by use of antagonistic fungi, plant resistance and neem

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ABSTRACT

Simultaneous infestation with root-knot nematodes (RKN) and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) leads to formation of a disease complex that increases crop losses than effect of either RKN or FOL. In this study a management programme involving plant resistance, biological control agents, and neem was carried out to manage RKN and fusarium wilt disease complex. The biological control agents were *Purpureocillium lilacinum* (PL) and *Trichoderma harzianum* (TH) while the RKN was *Meloidogyne javanica*. *In vitro* dual culture plates were set up to test the interaction of biological control agents and FOL. Greenhouse experiments were conducted using two tomato cultivars Rambo F1 and Prostar F1. The treatments were; PL, TH, PL–TH, neem, PL neem, TH neem, and PL–TH neem. Each treatment was replicated four times and the treatments set up in a randomised complete block design in the greenhouse. Inhibition of FOL mycelial growth by TH and PL was 51.9%, and 44% respectively by the ninth day *in vitro* culture plates. In the cultivar, Prostar F1, the treatments PL–TH, PL, and TH in the presence or absence of neem had a FOL disease severity score significantly lower than the untreated control. Host resistance sufficed to prevent infection of Rambo F1 with FOL. The treatments PL–TH, PL and TH reduced FOL propagules and *M. javanica* juveniles in the roots and performed even better when combined with neem in both tomato cultivars. Therefore, a host that is resistant combined with biological control agents and organic amendments can be used in the management of RKN and FOL in tomato production.

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Antagonistic fungi; fusarium wilt; neem; *Paecilomyces lilacinus*; root-knot nematodes; *Trichoderma harzianum*

1. Introduction

Fusarium oxysporum f. sp. *lycopersici* (Snyder and Hansen) pathogen causes yield losses to the tomato crop and the losses are further increased by simultaneous infestation with root-knot nematodes (RKN). Integrated disease control incorporating biological control agents, organic amendments, host resistance would be an alternative to chemicals that have a negative effect on the environment. Among the most researched biological control agents are fungal agents like *Paecilomyces lilacinus* (Thom) Samson syn. *Purpureocillium*

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lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (Luangsa-ard et al., 2011). *Paecilomyces lilacinus* parasitises female nematodes and also reduces hatching of nematode eggs (Pau et al., 2012), and reduces the number of juveniles in the soil.). *Trichoderma harzianum* has also been widely used in the control of plant pathogens and nematodes. The effect of TH in parasitising eggs (Naserinasab, Sahebani, & Etebarian, 2011) and second stage juveniles of RKN (Dababat & Sikora, 2007), and inhibiting fungal pathogens have been reported by several authors (Fatima, Noureddine, Jamal, Henni, & Mabrouk, 2015; Rehman, Lawrence, Kumar, & Badri, 2012).

Tomato cultivars with resistance to fusarium wilt and RKN have been developed. Use of resistant cultivars is one of the most effective methods of controlling fusarium wilt disease (Oliveira et al., 2013). Tomato cultivars that are resistant to RKN have the Mi gene, that confers resistance to *Meloidogyne incognita*, *M. javanica* and *M. arenaria* (Verdejo-lucas, Cortada, Sorribas, & Ornat, 2009). Organic amendments such as animal manures and composts have been known to increase agricultural productivity and improve plant health (Bonilla, Gutiérrez-barranquero, Vicente, & Cazorla, 2012). Neem is a common organic amendment that provides nutrients and control nematodes (Lokanadhan, Muthukrishnan, & Jeyaraman, 2012).

The study was carried out to investigate the effect of using disease resistance, biological controls and an organic amendment in the management of RKN (*M. javanica*) and FOL pathogen in two tomato cultivars Rambo F1 and Prostar F1. In a previous study, Rambo F1 was found to be resistant to the isolate of FOL while Prostar F1 was found to be very susceptible to the same isolate. The two tomato cultivars Prostar F1 and Rambo F1 were used in tomato production in Mwea West Sub County, Kenya (Mwangi, Kimenju, Narla, Kariuki, & Muiru, 2015).

2. Materials and methods

2.1 Interaction of biocontrol fungi and F. oxysporum f. sp. lycopersici in dual culture plates

The interactions between the fungi FOL, PL and TH were studied by dual culture techniques as described by Siameto, Okoth, Amugune, and Chege (2010). This was to pre-test antagonism of biological control agents against FOL and at the same time test compatibilities of TH and PL. Five-day old culture plates of FOL, PL and TH were used in these experiments. To study the antagonistic activity between FOL and PL; a plate of PDA was inoculated with five-millimetre mycelia disc of FOL and was placed 10 mm from the end of a 90 mm diameter petri-dish. A five-millimetre mycelia disc of PL was placed on the same PDA plate opposite to FOL mycelia disc. The FOL fungus was then placed separately at the centre of another PDA plate as control. Each set was then replicated four times and was set in a completely randomised design on the laboratory bench. The culture plates were incubated at 24 ± 2°C for 12 days. Colony growth of FOL fungi was observed and its radial growth towards the biocontrol fungi in the dual culture plates recorded every day. Radial growth in the control experiment was also recorded. Other dual culture tests: FOL/TH and PL/TH were set up in a similar manner. The control for FOL/TH test was a mycelial disc of FOL fungus placed separately on a PDA agar plate. In the PL/TH test, controls were mycelia discs of PL and

TH that were set up separately in different PDA agar plates. Mycelia discs of TH were only placed after three days because of its very high growth rate. This experiment was repeated twice.

2.2. Greenhouse experiment

A greenhouse study was done at the University of Nairobi, College of Agriculture and Veterinary Sciences (CAVS), Upper Kabete Campus field station using the tomato cultivars Prostar F1 and Rambo F1. Ambient day temperatures ranged from 21°C to 34°C in the greenhouse during the period of study.

2.3. Preparation of F. oxysporum f. sp. lycopersici inoculum and inoculation of tomato plants

The isolate of FOL was obtained from infected tomato plants. The identity was further confirmed by carrying out pathogenicity tests using tomato cultivar Money Maker. The closest match of the isolate (100% similarity) was with FOL in the MLST (http://www.cbs.knaw.nl/Fusarium) database. The isolate has been given a gene bank accession number MH587166.

The isolate of FOL, accession number MH587166 was grown on PDA for five days at room temperature (24°C ± 2). Two PDA cores of fungal growth were obtained by use of a cork borer (4 mm) and were used to inoculate 100 ml czapek dox growth medium put on a rotatory shaker at 50 rates per minute (rpm) at room temperature. After one week, the mycelia were harvested by sieving using a sterilised tea strainer and was used to inoculate one kilogram of sterilised sand-maize meal medium (900 g sand,100 gm maize flour, 200 ml water) in a transparent three kilograms polythene bag. After two weeks, one hundred grams of the fungal growth on the surface was scraped using a sterilised spatula. This was mixed with 900 ml of water agar (0.001%) in a one-litre media bottle and was shaken vigorously. The suspension was then filtered using a muslin cloth folded four times. The spore count in the filtrate was estimated by serial dilution of the filtrate and plating. The filtrate was centrifuged at 2250 rate per minute for 10 min, and the top layer siphoned out by means of a pipette and then adjusting to a concentration of 1×10^7 spores per ml by adding sterile distilled water. Inoculation was performed by drenching each pot with the fungal suspension, at the rate of 1×10^7 spores per gram of soil.

2.4. Preparation of second stage (J2s) inoculum and inoculation of tomato plants

The RKN (*M. javanica*) was obtained from Dr. Kariuki from the Department of Agricultural Science and Technology, Kenyatta University. Second stage juveniles (J2s) were extracted from Cal J tomato roots infested with *M. javanica* using a modification of a method of extraction described by Coyne, Nicol, and Claudius-Cole (2007). A hole was made two centimetres near each plant using a plastic spoon. Thirty millilitres of suspension contained 2000 J2s was dispensed into the holes which were then covered.

2.5. Source, preparation and inoculation of P. lilacinus and T. harzianum inocula

P. lilacinus was obtained from a commercial product Bio-Nematon$^{(TM)}$ 1.15WP [*P. lilacinus* 1.15%WP (1×10^8 cfu/g)]. Inoculation was performed by drenching each pot with the fungal suspension at the rate of 1×10^7 spores per gram of soil. The TH (Trianum, strain T-22:1×10^9 cfu/g by Koppert Biological Systems) was applied as a drench by dissolving 2.5 g in 250 ml water.

2.6. Planting of the seedlings, application of the treatments and experimental design

Three-week old tomato seedlings of the tomato cultivar Rambo F1 and Prostar F1 were transplanted separately into plastic pots (14 cm diameter and 8 cm height) filled with one kg sterilised sand and soil mixture. The tomato cultivars were purchased from agrochemical shops in Nairobi. The treatments were; PL, TH, PL–TH, neem, PL neem, TH neem, PL–TH neem and two controls. One control application was of Carbendazim 500 WP (Bavistin^{TM}) while the other was a negative control. Carbendazim is a systemic fungicide to control FOL pathogen but was also used as positive chemical control of RKN because it was found to control nematodes in this investigation. In the negative control, only FOL and *M. javanica* J2s were inoculated.

The neem imported from India was applied at the rate of 20 g per plant. In the control experiment, 40 ml of 0.25% Carbendazim was applied. The applications of the treatments to experimental plants were done four days after infesting with FOL inoculum and *M. javanica* J2s. The treatments were replicated four times and were arranged in a randomised block design in the greenhouse, and the experiment repeated twice. The plants were watered daily.

2.7. Data collection and analysis

The Data on *in vitro* culture plates obtained by measuring mycelia growth was analysed by paired *T*-tests using GenStat statistical package (Discovery Edition 14). The test plants were harvested eleven weeks after transplanting and dry shoot weight and total weight of the fruits recorded. The method of obtaining J2s from the roots is as described by Whitehead and Hemming (1965), while FOL propagules were obtained in terms of colony forming units per gram of root by dilution plate technique. Disease severity was assessed by modification of a wilting index by Akram, Anjum, and Ahmad (2014) at the time of harvesting the plants. The severity scale 1 represents no symptom, 2 = wilting and yellowing covered less than 25%, 3 = wilting and yellowing was less than 50%, 4 = wilting and yellowing was more than 50%.

Data was analysed by ANOVA, using GenStat statistical package (Discovery Edition 14). Counts of J2s was normalised before analysis by logarithmic transformation (Log base 10), while data on number of FOL propagules was normalised by square root transformation. Means of non transformed data was compared using Least Significant Difference (L.S.D.) at 5% level of significance and were separated using Fisher's Protected L.S.D. test. Means of transformed data was compared using L.S.D. at 5% level of significance.

3. Results

3.1 Antagonism of T. harzianum and P. lilacinum against F. oxsporum f. sp. lyopersici in vitro

T. harzianum and PL showed antagonistic activity to FOL (Table 1 and Figures 1 and 2). Paired *T*-sample tests results showed there were significant differences between mycelia growth of FOL in dual culture plate of FOL and TH, versus FOL control plate (Table 1). There were also significant differences in FOL mycelia growth between a dual culture plate of FOL and PL versus FOL control plate. The two biological control agents TH and PL were not significantly different in antagonism to each other *in vitro* conditions (Table 1).

3.2. Effect of T. harzianum, P. lilacinum and neem treatment in controlling M. javanica and fusarium wilt pathogen in tomato cultivars

Disease severity in the treatments, TH, PL and PL–TH was significantly ($P < .05$) lower than in the control though not significantly different from each other in cultivar Prostar F1. The performance of the biological agents was enhanced when neem was added such that the treatments, PL neem, TH neem and PL–TH neem, had a lower FOL disease severity score than their respective treatments without neem in the cultivar Prostar F1. The disease severity score in all treatments in cultivar Rambo F1 was not significantly different from the inoculated and untreated control (Figure 3).

The treatments, PL, TH, and PL–TH treatment had significantly ($P < .05$) lower number of *M. javanica* J2s and FOL propagules compared to the control in both tomato cultivars (Table 2). Neem, when combined with biological control agents enhanced their biological control ability such that the treatments, PL neem, TH neem and PL–TH neem had a significantly higher control of *M. javanica* J2s and FOL propagules compared, to their respective treatments without neem, in both tomato cultivars Prostar F1 and Rambo F1 (Table 2). The dry shoot weight and total weight of fruits were significantly higher in the PL neem, TH neem and PL–TH neem treatment compared to the respective treatments, PL, TH and PL–TH treatment in both tomato cultivars (Table 2).

4. Discussion

4.1 Management of M. javanica and F. oxysporum f. sp. lyopersici pathogen by T. harzianum and P. lilacinum and neem

The occurrence of an inhibition zone in dual culture plate of FOL pathogen and TH may be due to production of diffusible volatile and nonvolatile fungal metabolites such as

Table 1. Growth of fungal mycelia in dual culture plates compared to controls and results of paired *T*-sample tests.

	Mean mycelia growth in mm			
Dual culture vs control	Dual culture	Control	*T*-test statistic	*P*-value
FOL/TH vs FOL	1.86	3.9	6.69	<.001
FOL/PL vs FOL	2.3	3.9	5.01	<.001
TH/PL vs TH	3.9	4.5	0.99	.351
PL/TH vs PL	1.55	1.58	0.45	.62

Note: FOL: *Fusarium oxysporum* f. sp. *Lycopersici*; PL: *P. lilacinum*; TH: *T. harzianum*.

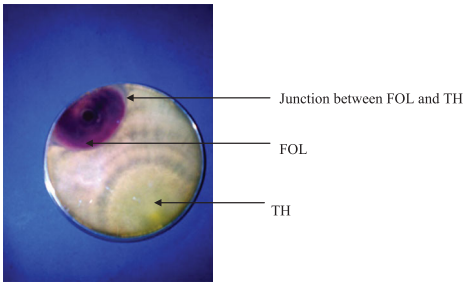

Figure 1. Antagonism between FOL and TH.

harzianic acids, tricholin, viridian and gliovir that prevent pathogen mycelia growth (Rini & Sulochana, 2007). Other researchers have reported on inhibition of FOL *in vitro* by TH (Javaid, Afzal, Bashir, & Shoaib, 2014; Kamali, Pourjam, & Sahebani, 2015).

The severity of fusarium wilt disease and number of *M. javanica* J2s decreased in the soil due to TH. *Trichoderma harzianum* in greenhouse experiments has been reported to be effective in suppressing Fusarium wilt pathogen in tomato plants (Selvakumar et al., 2014). Dababat and Sikora (2007) observed reduction in root galls in tomato plants. Application of TH significantly reduced population density of *Meloidogyne* spp. and the severity of Fusarium wilt (Kamali et al., 2015).

In vitro tests showed that PL was antagonistic towards FOL and inhibited mycelia growth, the evidence being a zone of inhibition between PL and FOL pathogen. The mode of antagonism was likely by production of fungal metabolites and not by a competitively high growth rate since radial mycelia growth of PL was less than that of FOL. There are very few reports of antagonism of PL towards fungal pathogens; however, Lan, Zhang, Zong, Ma, and Wang (2017) recorded high inhibitory activity against *Rhizoctonia solani*, and *Verticillium dahlia in vitro*. Under *in vivo* conditions, the number of FOL fungal propagules in the soil was reduced in both tomato cultivars Rambo F1 and Prostar F1 due to application of PL. It also lowered total *M. javanica* J2s populations in the roots and soil. *Paecilomyces lilacinus* destroys nematodes eggs by producing chitinases that inhibit eggs development (Chan, Cai, Taylord, Chan, & Yeha, 2010). There are many reports on mycoparasitism of nematode eggs by PL resulting in reduction of nematode multiplication in the soil (Kannan & Veeravel, 2012; Nagesh, Hussaini, Ramanujam, & Chidanandaswamy, 2006).

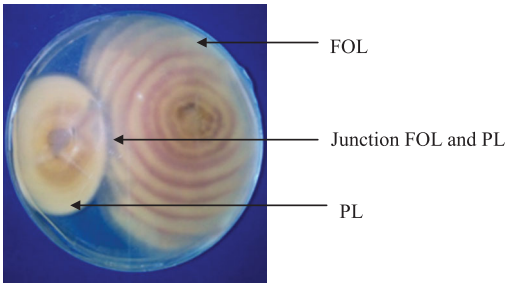

Figure 2. Antagonism between PL and FOL.

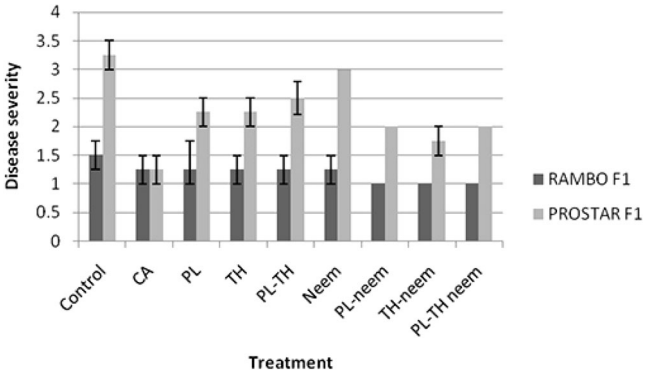

Figure 3. Effect of different treatments on fusarium wilt disease severity in FOL and root-knot disease complex in tomato cultivars Rambo F1 and Prostar F1 in greenhouse environment. Means were compared using LSD = 0.6. The I bars signify 5% error. FOL: *Fusarium oxysporum* f. sp. *lycopersici*; PL: *P. lilacinum*; TH: *T. harzianum*.

The two biological control agents, TH and PL were not found to have an inhibitory effect on each other under *in vitro* conditions. The expectation that PL–TH treatment being a combined application would have a combined effect of both PL and TH was not observed, neither did PL–TH neem have a combined effect of both PL neem and TH neem. Experiments on effectiveness of a combined treatment of PL and TH in the control of FOL and RKN have been reported (Nagesh et al., 2006). So far, there are no reports of antagonism between PL and TH. The lack of synergism or additive effect

Table 2. Effect of different treatments on *F. oxysporum* f. sp. *lycopersici*, *M. javanica* juveniles and growth in tomato cultivars Prostar F1 and Rambo F1.

	Treatment	*M. javanica* J2s	CFU of FOL/ gm of root	DSW	Fruit weight in (g)
Prosta F1	Control	4663 (3.67)	400 (20)	15.4a	85.8a
	Carbendazim	1167 (3.06)	150.3 (12.4)	20.73cd	102.7ab
	PL	2753 (3.44)	161 (12.7)	19.5bc	114.7bc
	TH	3290 (3.52)	14.3 (3.4)	18.93bc	115.3bc
	PL–TH	2270 (3.35)	7.7(2.2)	17.23ab	104.7ab
	Neem	3619 (3.56)	453.3 (21.3)	25efg	113.0bc
	PL neem	1720 (3.35)	40 (6.3)	27.6ghi	136.7de
	TH neem	2253 (3.35)	10 (3.2)	25.7efg	133.0cde
	PL–TH neem	1503 (3.17)	11 (3.3)	28.6hi	138.7e
Rambo F1	Control	4435 (3.65)	300 (17.3)	18.3abc	91.1a
	Carbendazim	1328 (3.11)	153 (12.4)	23.8def	116.8bcd
	PL	2783 (3.44)	116 (10.8)	24de	117.4bcd
	TH	3028 (3.48)	13 (3.6)	23.3b	122.2bcde
	PL–TH	3010 (3.48)	10 (3.2)	24def	119.6bcde
	Neem	2952 (3.47)	286 (16.9)	27ghi	205.9g
	PL neem	1953 (3.29)	14 (3.7)	32.2j	202.4g
	TH neem	2041 (3.31)	2.5 (1.6)	28.5hi	197.3fg
	PL–TH neem	1800 (3.25)	1.9 (1.4)	29.7ij	180f
	L.S.D.	0.10	3.0	3.3	20.3

Notes: Data are mean of four replicates. Means followed by the same letter in the same column are not significantly ($P < .05$) different according to Fisher's L.S.D test. J2s: stage two juveniles; FOL: *F. oxysporum* f. sp. *Lycopersici*; PL: *P. lilacinum*; TH: *T.harzianum*; DSW: dry shoot weight; CFU: Colony Forming Units; control: inoculated with *M. javanica* and FOL but not treated. Values in brackets are [1]$\log_{10}$ and [2] square root transformed means and used in comparison with L.S.D. Table includes data previously presented in Mwangi, Muiru, Narla, Kimenju, and Kariuki (2018). Copyright by Taylor and Francis Group.

when the two were combined in the control of FOL and RKN might suggest that in soil, there was one fungus inhibiting the other. Boer, Van der Sluis, Van Loon, and Bakker (1998) reported that antagonism between introduced biological control agents can be detected if effect of application of a combination is zero, or similar to one of the biological control agents. In this study, the two fungi had a similar mode of action in that both were antagonistic to FOL and produced zones of inhibition in dual culture agar plates. However, compatibility tests of the two fungi in dual agar plates showed they were compatible and did not cause any significant inhibition against each other. The two fungi could have acted against each other by mycoparasitism. This was not tested in this experiment. Future tests would require pre-testing a combination of biocontrol fungi for mycoparasitic activity against each other.

Neem treatment controlled *M. javanica* J2s in both tomato cultivars. Neem cake powder has also been used in the greenhouse and field conditions in order to control nematodes by producing azadirachtin and other chemicals which inhibit larval growth (Lokanadhan et al., 2012). Neem has also been reported to control FOL (Kimaru, Waudo, Monda, Seif, & Birgen, 2004; Nagesh et al., 2006). In this experiment, application of neem treatment alone did not control the FOL wilt pathogen or reduce the FOL propagules in the roots of both tomato cultivars. The mode of action by neem could have been providing nutrients and in the absence of antagonistic fungi, FOL fungal pathogen multiplied. The implication of lack of control of FOL by application of neem as seen in this study is that, if used alone in the control of any fungal pathogen, then pretesting has to be done to elucidate its mode of action otherwise the end result might be an increase in the pathogen. Neem increased the biological control of FOL and *M. javanica* J2s when applied together with the biological control agents. Neem as an organic amendment could have enhanced the activity of biological control agents by providing a Carbon to Nitrogen ratio that is optimal for fungal growth (Lamour, Van den Bosch, Termorshuizen, & Jeger, 2000).

The effect of host resistance sufficed to prevent infection of Rambo F1 with FOL. In applications with neem either alone or combined with biological control agents, the effect of host resistance on growth was evident because of a higher weight of fruits in the cultivar Rambo F1 than in Prostar F1 in those applications. The inherent ability of host resistance worked well in the presence of neem in cultivar Rambo F1 to enhance yield.

The effectiveness of PL neem and TH neem in the control of *M. javanica* and FOL points to the fact that either of them can be applied in tomato production. The implication is that if applied with a resistant cultivar they would keep populations of RKN and FOL pathogen low preventing build up that normally results in emergence of resistant strains. Therefore host resistance, application of biological control agents and organic amendments would be an important strategy for the control of RKN and FOL in tomato production.

Disclosure statement

No potential conflict of interest was reported by the authors.

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