PAPAYA SEEDLINGS COLONIZED BY THE BIO-AGENTS *TRICHODERMA HARZIANUM* AND *PSEUDOMONAS FLUORESCENS* TO CONTROL ROOT-KNOT NEMATODES

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Summary. Experiments were conducted to produce seedlings of papaya with roots colonized by *Trichoderma harzianum* and *Pseudomonas fluorescens* for the control of the root-knot nematode *Meloidogyne incognita*. Seed treatment with *P. fluorescens* (10^8 cfu/g) and application of *P. fluorescens* and *T. harzianum* (10^6 cfu/g), each at 5 g/kg soil, reduced significantly the eggs/egg-mass of *Meloidogyne incognita*. Nursery soil treated with formulations of either *P. fluorescens* or *T. harzianum* was not significantly effective in reducing the nematode root-galling index. However, root-knot nematode control was improved when both bio-agents were used in the nursery soil at a rate of 5 g/kg each. Combined use of *P. fluorescens* and *T. harzianum* did not affect root colonization by either bio-agent. Seed and soil treatment with the formulations of the bio-agents produced highly vigorous seedlings with significantly increased growth. The seedling roots were colonized by both the bio-agents, which, therefore, would be carried to the field on transplanted seedlings. Thus, the results outline the potential for the production of papaya seedlings with roots colonized by combined formulations of *P. fluorescens* and *T. harzianum* for the control *M. incognita* under field conditions.

Key words: *Carica papaya*, *Meloidogyne incognita*, seed treatment.

The root-knot nematode (RKN) *Meloidogyne incognita* (Kofoid *et* White) Chitw. greatly reduces the productivity of papaya (*Carica papaya* L.) (McSorley, 1981; Reddy e*t al*., 1988; Reddy and Khan, 1991). Surveys made in papaya growing areas of south India revealed very high incidences of *M. incognita* (Reddy *et al.,* 1990). Moreover, in India, papaya seedlings are often infected with *M. incognita*. Infected seedlings will result in dissemination of the parasite to the main field and poor crop stand and yield. As the use of chemical nematicides can be hazardous, the use of formulations of biocontrol agents, such as *Trichoderma harzianum* Rifai and *Pseudomonas fluorescens* Migula, would be an alternative for the production of healthy seedlings of papaya. *Pseudomonas fluorescens* is effective against root-knot and other nematodes (Santhi and Sivakumar, 1995; Perveen *et al.*, 1998; Siddiqui *et al.*, 1999; Rao *et al.*, 2002a, b) and *T. harzianum* against root-knot and citrus nematodes (Reddy *et al*., 1996; Rao *et al*., 1997, 1998). Also, synergistic effects of bio-agents in combined treatments have been reported (Kerry, 2000; Siddiqui and Shaukat, 2004). Therefore, the objective of these investigations was to standardize a method of producing bio-agent colonized seedlings of papaya using *P. fluorescens* and *T. harzianum*, which would help in the management of root-knot nematodes in papaya both in the nursery and in the field. It was hypothesized that the seedlings with roots colonized by the bio-agents would carry the bio-agents to the field, which, in turn, would enrich the field soil with bio-agent propagules within three or four growing seasons.

MATERIALS AND METHODS

The local isolates of *P. fluorescens* (IIHR-PF 2) and *T. harzianum* (IIHR-TH 2) are maintained in a collection at the Indian Institute of Horticultural Research. They are mass produced through liquid and solid fermentation processes (the details of the fermentation process are not revealed here for patent considerations). The formulations used in this experiment were produced at Indian Institute of Horticultural Research, Bangalore, India, and contained *P. fluorescens* (10^8 spores/g) and *T. harzianum* (10^6 spores/g).

The experiment was conducted during two growing periods: Season 1 - July-September 2004, and Season 2 - June-September 2005. We followed the procedures used by nursery men in India for the production of seedlings of papaya suitable for transplanting. The nursery soil (mixture of soil, farmyard manure and sand in the ratio of 3:2:1) contained 94 second-stage juveniles (J2) of *M. incognita* per 100 g of soil. The soil was mixed with the bio-agent formulations and polythene bags (10 × 15 cm) were filled with one-kg aliquots of the mixture. Then, single seeds of papaya (cv. Arka surya) were sown in each bag. The treatments (Tables I-IV) were: *a*) seed treatment with *P. fluorescens* at 25 g/kg seeds; *b*) nursery soil mixed with the formulation of *P. fluorescens* at 5 g/kg; *c*) nursery soil mixed with the for-

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mulation of *T. harzianum* at 5 g/kg; *d*) nursery soil mixed with the formulations of *P. fluorescens* and *T. harzianum*, each at 5 g/kg; *e*) seed treatment with *P. fluorescens* at 25 g/kg and nursery soil mixed with the formulation of *P. fluorescens* at 5 g/kg; *f*) seed treatment with *P. fluorescens* at 25 g/kg and nursery soil mixed with the formulation of *T. harzianum* at 5 g/kg; *g*) seed treatment with *P. fluorescens* at 25 g/kg and nursery soil mixed with the formulations of *P. fluorescens* and *T. harzianum*, each at 5 g/kg; *h*) control with no treatment. Each treatment was replicated ten times in a completely randomized design.

Observations on the length of seedlings (from the lowest root tip to the tip of the top leaf), seedling weight, the root-knot index on a 1-10 scale (Bridge and Page, 1980), number of eggs/egg-mass, root colonization by *P. fluorescens* and *T. harzianum*, suppression of hatching of eggs by *P. fluorescens* and *T. harzianum*, root and soil nematode population densities were recorded 90 days after sowing.

Each root system of the ten seedlings was carefully washed to remove soil and excess water was removed using blotting paper. To evaluate root colonization by *P. fluorescens* and *T. harzianum*, root systems of five seedlings were weighed and cut into small pieces about 1 cm long. Two grams of root pieces were picked at random from each seedling: 1 g was taken to estimate root colonisation by *P. fluorescens* and 1 g was used to evaluate root colonization by *T. harzianum*, using the semi-selective media developed by King *et al*. (1954) and Papavizas and Lumbsden (1982), respectively. Petri plates were incubated at 27-29 °C for 15 days. Soil propagule densities of both bio-agents were estimated by following a serial dilution technique and using the above-mentioned semi-selective media.

To determine the number of RKN eggs/egg mass, two egg masses of *M. incognita* were randomly selected from each of the remaining five plants. The egg masses were dissolved separately in 0.05% sodium hypochlorite solution (Hussey and Barker, 1973) and the number of eggs in each was counted. To assess the effect of *P. fluorescens* or *T. harzianum* on suppression of hatching of eggs, two egg masses were again randomly selected from each of the five plants. They were surface sterilized, separately, with 0.01% sodium hypochlorite in a Petri plate for 30 seconds and then placed in a Petri plate (5-cm-diam.) containing 5 ml of sterile distilled water, and incubated at 27 °C. The numbers of J2 emerged were recorded at intervals of 24 hours for five consecutive days and per cent suppression of egg hatching was calculated.

Trichoderma harzianum was isolated from five egg masses of *M. incognita* collected, separately, from five plants by using the semi-selective medium mentioned above.

Root populations of RKN were estimated from 10 g samples of roots from each plant. The root samples were stained using acid fuchsin following the method of Bridge *et al.* (1982), homogenised, and the numbers of nematodes in the roots were recorded. To estimate the soil population density of the nematode, the J2 of *M. incognita* were extracted from 100 cm^3 soil per replicate by Cobb's sieving and decanting method (Cobb, 1918) and counted.

The data were analyzed following procedures described by Little and Hills (1978).

Table I. Effects of treatments with *Trichoderma harzianum* and *Pseudomonas fluorescens* on the growth of papaya seedlings in a nursery infested with *Meloidogyne incognita*.

Treatment	Seedling height (cm)		Seedling weight (g)		Root galling index (1 – 10)	
	Season 1	Season 2	Season 1	Season 2	Season 1	Season 2
Seed treatment (st) with *P. fluorescens* (Pf) (Pf – st)	24.3	26.3	4.4	5.0	6.5	6.8
Nursery soil mixed with *P. fluorescens* at 5 g/kg	26.5	25.2	5.8	6.1	5.5	6.2
Nursery soil mixed with *T. harzianum* at 5 g/kg	21.6	20.5	3.7	4.6	6.8	7.2
Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	27.9	25.6	6.6	6.0	4.6	4.2
Pf – st + Nursery soil mixed with *P. fluorescens* at 5 g/kg	30.5	29.3	7.2	6.8	3.4	4.2
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg	23.5	25.4	5.3	5.7	5.2	4.6
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	32.4	30.8	8.4	7.9	3.3	2.8
Control	19.7	17.5	3.2	4.1	8.4	8.2
C.D. (P = 0.05)	3.78	3.24	1.78	1.18	1.28	1.14

C.D. = Critical Difference

RESULTS AND DISCUSSION

Soil treatment with formulations of *P. fluorescens*, *T. harzianum* and a combination of these two bio-agents significantly increased the growth of the seedlings of papaya (Table I). These treatments also significantly reduced the root galling index in first and second season (Table I), and eggs/egg mass (Table II). There are several reports on the bio-control potential of *Pseudomonas* spp. (Santhi and Sivakumar, 1995; Perveen *et al.*, 1998; Siddiqui *et al.*, 1999; Rao *et al.*, 2002a, b) and *T. harzianum* (Reddy *et al.*, 1996; Rao *et al.*, 1997, 1998). The strain of *T. harzianum* colonised the egg masses of *M. incognita* and suppressed egg hatch. However, hyphae of this bio-agent were not detected inside the eggs, but *T. harzianum* was isolated from the egg masses of *M. incognita*. It was not possible to isolate *P. fluorescens* from the females or egg masses of *M. incognita* but hatching of eggs from egg masses isolated from seedlings treated with *P. fluorescens* was significantly less than in the control (Table III). Suppression of egg hatching in both cases was probably due to toxins released by the two bio-agents. Siddiqui and Shaukat (2004) reported that application of *P. fluorescens* and *T. harzianum* to unsterilized sandy loam soil greatly reduced population densities of *M. javanica* in the roots of tomato. Their work is significant in demonstrating the synergistic effect of *T. harzianum* on the production of nematicidal compound(s) by *P. fluorescens*. These authors documented overwhelming expression of the *phl'-' lacZ* reporter gene by *P. fluorescens* when the medium was amended with culture filtrate of *T. harzianum*. This gene encodes for the production of the antimicrobial 2,4-DAPG (2,4-diacetylphloroglucinol). Kerry (2000) has reported rhizospheric interactions of *Paecilomyces lilacinus* and *Pochonia chlamydosporia* in soil infested with RKN.

Seed treatment with *P. fluorescens* increased the height of the seedlings significantly (Table I). The role of *P. fluorescens* on plant growth promotion is well doc-

Table II. Effects of treatments with *T. harzianum* and *P. fluorescens* on root and soil populations of *M. incognita*.

Treatment	Juveniles and adults/ 10 g roots		J2/100 cm^3 soil	
	Season 1	Season 2	Season 1	Season 2
Seed treatment (st) with *P. fluorescens* (Pf) (Pf – st)	47	52	78	84
Nursery soil mixed with *P. fluorescens* at 5 g/kg	42	46	70	66
Nursery soil mixed with *T. harzianum* at 5 g/kg	50	48	65	72
Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	35	37	52	55
Pf – st + Nursery soil mixed with *P. fluorescens* at 5 g/kg	38	42	56	62
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg	45	40	63	55
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	31	26	45	31
Control	68	74	126	142
C.D. (P = 0.05)	6.6	7.4	8.5	9.7

C.D. = Critical Difference; J2 = second-stage juveniles

Table III. Effects of treatments with *T. harzianum* and *P. fluorescens* on eggs/egg mass and suppression of hatching of eggs of *M. incognita*.

Treatment	Number of eggs/egg mass		% suppression of hatching of eggs	
	Season 1	Season 2	Season 1	Season 2
Seed treatment (st) with *P. fluorescens* (Pf) (Pf – st)	389	380	32	28
Nursery soil mixed with *P. fluorescens* at 5 g/kg	364	358	45	42
Nursery soil mixed with *T. harzianum* at 5 g/kg	375	389	43	40
Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	358	326	65	67
Pf – st + Nursery soil mixed with *P. fluorescens* at 5 g/kg	326	338	55	52
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg	347	385	54	62
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	310	304	67	74
Control	412	404	0	0
C.D. (P = 0.05)	33.2	28.7	7.4	5.7

C.D. = Critical Difference

Table IV. Effects of treatments with *T. harzianum* and *P. fluorescens* on root colonization of papaya seedlings.

Treatment	Root colonization by *T. harzianum* (CFU/g)		Root colonization by *P. fluorescens* (CFU/g)	
	Season 1	Season 2	Season 1	Season 2
Seed treatment (st) with *P. fluorescens* (Pf) (Pf – st)	0	0	8769	6548
Nursery soil mixed with *P. fluorescens* at 5 g/kg	0	0	18457	19689
Nursery soil mixed with *T. harzianum* at 5 g/kg	22649	21632	0	0
Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	21895	21254	18249	18954
Pf – st + Nursery soil mixed with *P. fluorescens* at 5 g/kg	0	0	24531	22387
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg	22412	22158	8566	7569
Pf – st + Nursery soil mixed with *T. harzianum* at 5 g/kg and *P. fluorescens* at 5 g/kg	20874	21549	24124	21863
Control	0	0	00	0
C.D. (P = 0.05)	2365.7	2138.4	2487.2	2348.8

C.D. = Critical Difference; CFU = Colony Forming Units.

umented (Shouan Zhang *et al.*, 2003; Kishore *et al.*, 2003). However, in our investigation *P. fluorescens* alone was not sufficient to control RKN nematode infestation on the seedlings of papaya as it only reduced nematodes in roots and soil by 40-50% (Tables I and II). This could be due to the low rate of application resulting in a low level of root colonization (Table IV) and, therefore, in low hatching suppression of eggs of *M. incognita* (Table III).

Treatment of seeds with *P. fluorescens* and of the soil with both *P. fluorescens* and *T. harzianum* was significantly more effective than all other treatments in reducing root-galling index on papaya seedlings and root and soil populations of *M. incognita* (Tables I and II). They also increased growth components of the seedlings and suppressed egg hatching (Tables I and III), especially when seed treatment with *P. fluorescens* was combined with soil treatment with both bio-agents.

The roots of the seedlings were colonized by both bio-agents (Table IV). Root colonization is an important criterion for assessing the bio-efficacy of any formulated product, as transplanted seedlings would then carry the bio-agents to the field. The combined use of *P. fluorescens* and *T. harzianum* did not affect the colonization of roots by either bio-agent (Table IV).

The role of RKN in increasing wilt disease caused by *Fusarium solani* (Mart) Sacc., on papaya is established (Khan *et al.,*1992). Our results have practical implications as the combination of these two bio-agents has a distinct advantage, and they have also been found helpful in the management of *Fusarium* wilt of papaya (Rao, 2003). Thus, the development of a formulation with both *P. fluorescens* and *T. harzianum* is likely to be very useful.

In India, most of the nurseries are infested with RKN. This investigation demonstrated that, under such conditions, the use of bio-agents may not be sufficient to produce seedlings of papaya free of RKN. Nevertheless, it has been demonstrated that roots colonized by *P. fluorescens* and *T. harzianum* do suppress RKN and improve growth of papaya. The extent to which the use of such colonized papaya seedlings may control RKN needs to be ascertained under Indian local conditions.

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