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Biological control of cowpea [*Vigna unguiculata* (L.) Walp.] root-rot caused by *Macrophomina phaseolina* (Tassi.) Goid. by bacterial and fungal antagonists

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ABSTRACT: **Seven bacterial isolates (four of *Pseudomonas fluorescens* and three of *Bacillus subtilis*) and seven isolates of fungal antagonists (six of *Trichoderma* spp. and one of *Lentinus* spp.) were tested for their ability to inhibit cowpea root-rot pathogen, *Macrophomina phaseolina* under both *in vitro* and *in vivo* conditions. Among the bacterial antagonists, *P. fluorescens charvae* isolate recorded a maximum inhibition of mycelial growth of the pathogen followed by *P. fluorescens* isolate 2, whereas in fungal antagonists, *T. koningii* recorded a maximum inhibition followed by *L. edodes* and *T. viride* isolate 4. Pot-culture evaluation, under greenhouse conditions using *P. fluorescens charvae*, *T. koningii* and *L. edodes* revealed talc based powder formulation of *P. fluorescens charvae* and *T. koningii* was found to be the best in controlling root-rot incidence of cowpea.**

KEY WORDS: *Bacillus subtilis*, biological control, cowpea, *Pseudomonas fluorescens*, *Trichoderma* spp.

INTRODUCTION

India is the major pulses growing country of the world, accounting for roughly one third of the total area under pulses and one fourth of the total production (Pandey and Singh, 2001). Among the pulses, Cowpea [*Vigna unguiculata* (L.) Walp.] is considered to be versatile pulse crop serving as vegetable, fodder and cover crop. It is grown throughout the tropics and sub-tropics. In the world, cowpea is grown annually in an area of 5 million hectares (Ferry, 1981). *Macrophomina phaseolina* (Tassi.) Goid. is an important soil-borne plant pathogen causing root-rot on cowpea (Sinha and Khare, 1977). The pathogen is both seed and soil inhabitant in nature. Protection of crop plants from diseases using fungicides has been the regular practice for many years and it is a widely adopted strategy and has been yielding a phenomenal result in reducing the disease severity, but *Macrophomina phaseolina* is reported to have developed resistance to some of the fungicides (Anil Kumar and Sastry, 1979). Biological control of plant pathogens using antagonistic microorganisms is eco-friendly, economical and efficient method and can be successfully exploited in the framework of integrated disease management. In the present investigations, the role of *Trichoderma* spp., *B. subtilis* and *P. fluorescens* in root-rot management is ascertained.

MATERIALS AND METHODS

Isolation of pathogen

Cowpea plants (Cultivar Co-4) showing typical root-rot symptoms were collected from farmers' fields at different places in Tamil Nadu (Table 1). The pathogen was isolated by tissue segment method (Rangaswamy, 1972) on potato dextrose agar (PDA) medium. Axenic cultures of isolates of the pathogen were obtained by single hyphal tip method (Rangaswamy, 1972).

Isolation of fluorescent pseudomonads and *Trichoderma* spp. from the rhizosphere soil

Fluorescent pseudomonads and *Trichoderma* spp. were isolated from the rhizosphere soil by following serial dilution plate technique (10^{-4}, 10^{-5} and 10^{-6} dilution, respectively) (Warcup, 1950) by using King's B medium (King *et al.*, 1954) and *Trichoderma* selective medium (TSM), respectively.

Dual culture plate technique

The fungal antagonists and the pathogen (*M. phaseolina*) were cultured on PDA medium, while *P. fluorescens* and *Bacillus subtilis* were cultured on King's B medium and Nutrient agar medium, respectively. A 9 mm diameter disc of the each fungal antagonist was placed separately at one end of the sterilized Petri-plate containing 15 ml of PDA medium. A similar disc of the pathogen was placed on the medium at the opposite end. The linear growth of pathogen was measured at regular interval from 24 h after inoculation. For testing bacterial antagonists, the pathogen was inoculated at one end of the Petri-plates containing PDA medium. Each antagonist was streaked separately in the opposite end of the same Petri-plate. Each antagonist was replicated thrice and a suitable control was maintained without any antagonist. The mycelial growth of pathogen was measured 24 h after inoculation of bacterial antagonists. The results were expressed as percent growth reduction over control by using the following formula (Vincent, 1927). $I = C\text{-}T/C \times 100$ where, I is inhibition of fungal growth, C is fungal growth in control and T is fungal growth in the treatments.

Effect of antagonists on sclerotial production

In the above experiments, observation were recorded on sclerotial production 5 days after inoculation of pathogen. Three 9 mm discs containing the sclerotia of *M. phaseolina* were punched from each Petri-plate, separately. These three discs were placed separately in a beaker containing 10 ml of sterile distilled water and stirred for 30 minutes so as to separate the sclerotia from the medium. The contents were squeezed through cheese cloth and washed in several changes of sterile distilled water separately and transferred to glass vials containing 2.5 ml of 2.5 per cent ammonium sulphate. The sclerotia, which floated after 10 minutes, were filtered through a Whatman No. 42 filter paper separately and rinsed with the sterilized distilled water. The filter papers with these sclerotia were removed and the numbers of sclerotia were counted with a stereo-binocular microscope (Dhingra and Sinclair, 1978).

Greenhouse study

The effect of fungal and bacterial antagonists, alone or in different combinations on the management of root-rot of cowpea was studied under glasshouse conditions at Madurai (July, 2003) in completely randomized design with three replications. To conduct pot-culture experiments natural soil free from pathogenic microorganisms was used. The fungal and bacterial antagonists, found to inhibit significantly the growth of *M. phaseolina in vitro*, were selected for the experiment.

There were twenty treatments including control. Seed treatment with *P. fluorescens charvae* (10 g kg^{-1} of seed) and *T. koningii* (4 g kg^{-1} of seed) was carried out by using talc-based formulation of the antagonists. The talc-based formulation of *T. koningii* as well as *P. fluorescens charvae* was applied to the soil near root zone @ 2.5 kg ha^{-1} 20 DAS, separately, as per the treatment schedule. The bacterial inoculum (2.5 kg ha^{-1}) was mixed with 50 kg of soil and applied at the root region of the crop. The same method was also followed for soil application of *T. koningii*. Carbendazim was used as chemical check for seed treatment @ 2 g kg^{-1}

seed and as soil drench (0.1%) 20 DAS. Pots with addition of pathogen or without pathogen served as control. For each treatment three replications were maintained. The seed germination was recorded 5 DAS. Shoot length and disease intensity were recorded at 45, 60 and 75 DAS. Population of *Trichoderma* spp. and *Pseudomonas* spp. in the rhizosphere region was assessed separately (at 45, 60 and 75 DAS).

RESULTS AND DISCUSSION

Madurai isolate took two days for the initiation of the mycelial growth and the plate was completely covered (9 cm) in five days. In the case of other isolates, it took for three to four days for mycelial growth initiation and six to eight days for complete growth. The colour of the mycelium was grayish black in two isolates (Madurai and Coimbatore) and grayish white in the other three isolates (Killikulam, Namakkal and Rasipuram) (Table 2). Dhingra and Sinclair (1973) and Ghosh and Sen (1973) correlated the rate of mycelial growth of the isolates with their virulence on the different hosts and in the morphology of various isolates on *M. phaseolina* and the same was also reported by Pramella Devi and Singh (1998).

Inhibitory effect of bacterial and fungal antagonists against the pathogen using seven bacterial isolates (four isolates of *P. fluorescens* and three isolates of *B. subtilis*) tested against *M. phaseolina*, *P. fluorescens charvae* isolate recorded a maximum inhibition of 57.23 per cent over the

Table 1. Incidence of root-rot of cowpea in different areas of Tamil Nadu

Locality	District	Root-rot incidence (%)
1. Killikulam	Thirunelveli	46.50 (42.99)
2. Madurai	Madurai	61.20 (51.69)
3. Coimbatore	Coimbatore	46.20 (42.82)
4. Namakkal	Namakkal	48.60 (44.20)
5. Rasipuram	Namakkal	43.50 (41.27)
CD (P = 0.05)		1.76

Figures in the parentheses are arcsine-transformed values.

Table 2. Growth and morphological characters of *M. phaseolina* isolates

Isolates	Days taken for initiation of fungal growth	Mycelial growth 5 DAI (cm)*	Colour of the mycelium	Days taken for full growth
1. Killikulam (MP1)	3.0	8.64	Grayish white	7.0
2. Madurai (MP2)	2.0	9.00	Grayish black	5.0
3. Coimbatore (MP3)	3.0	8.79	Grayish black	6.0
4. Namakkal (MP4)	3.0	8.40	Grayish white	6.0
5. Rasipuram (MP5)	4.0	8.31	Grayish white	8.0
CD (P = 0.05)		0.05		

* Mean of four replications.

Table 3. Effect of bacterial antagonist on mycelial growth and sclerotial production of *M. phaseolina in vitro* (Dual culture Technique)

Antagonist	Mycelial growth at 3 DAI (mm)*	Inhibition over control (%)	Number of sclerotia / 9 mm disc
1. *P. fluorescens charvae*	38.50	57.23 (49.16)	67.50
2. *P. fluorescens* 1	56.40	37.34 (37.67)	91.75
3. *P. fluorescens* 2	51.50	42.78(40.85)	88.00
4. *P. fluorescens* (Native isolate 1)	58.50	35.00 (36.27)	94.25
5. *B. subtilis* 1	59.75	33.61 (35.43)	96.00
6. *B. subtilis* 2	66.40	26.22 (30.80)	99.25
7. *B. subtilis* 3	61.25	31.94 (34.41)	97.50
8. Control	90.00	-	151.80
CD (P=0.05)	2.73		3.94

* Mean of three replications

Figures in the parentheses are arcsine-transformed values.

DAI – Days after inoculation

Table 4. Effect of fungal antagonists on mycelial growth and sclerotial production of *M. phaseolina in vitro* (Dual culture Technique)

Antagonists	Mycelial growth (mm) at 5 DAI*	Inhibition over control (%)	Number of sclerotia/ 9 mm disc
1. *T. koningii*	21.75	75.84 (60.56)	43.57
2. *T. viride* 1	45.60	49.34 (44.62)	83.25
3. *T. viride* 2	35.80	60.22 (50.90)	71.75
4. *T. viride* 3	26.25	70.83 (57.31)	51.50
5. *T. viride* 4	23.50	73.89 (59.27)	46.50
6. *T. viride* (Native isolate1)	39.80	55.78 (48.32)	76.00
7. *L. edodes*	22.50	75.00 (60.00)	45.00
8. Control	90.00	-	148.75
CD (P=0.05)	1.73		2.34

* Mean of three replications

Figures in the parentheses are arcsine-transformed values.

DAI – Days after inoculation

Table 5. Efficacy of antagonists on the germination and disease incidence in pot- culture

Treatment	Germination (%)*	Root-rot incidence (%)* DAS			Disease reduction over control at 75 DAS (%)
		45	60	75	
1. ST with *P. fluorescens charvae* @ 10 g/ kg seed	93.34(75.0)	20.0(26.6	40.0(39.2)	46.7(43.1)	41.7(40.2)
2. T_1+ SA with *T. koningii* @ 2.5 kg/ha 20 DAS	93.3(75.0)	13.3(21.4)	26.7(31.1)	33.3(35.3)	58.3 (49.8)
3. T_1+ SA with *T. viride* 4 @ 2.5 kg/ha 20 DAS	93.3(75.0)	20.0(26.6)	33.3(35.3)	40.0(39.2)	50.0(45.0)
4. T_1+ SA with *P. fluorescens charvae* @ 2.5 kg/ha 20 DAS	100.0(90.0)	6.7(14.9)	20.0(26.6)	26.7(31.1)	66.7(54.7)
5. ST with *P. fluorescens* 2 @ 10 g/ kg seed	93.3(75.0)	26.7(31.1)	33.3(35.3)	46.7(43.1)	41.7(40.2)
6. T_5+ SA with *T. koningii* @ 2.5 kg/ha 20 DAS	93.3(75.0)	13.3(21.4)	26.7(31.1)	40.0(39.2)	50.0(45.0)
7. T_5+ SA with *T. viride* 4 @ 2.5 kg/ha 20 DAS	93.3(75.0)	26.7(31.1)	33.3(35.3)	40.0(39.2)	50.0(45.0)
8. T_5+ SA with *P. fluorescens* 2 @ 2.5 kg/ha 20 DAS	93.3(75.0)	20.0(26.6)	33.3(35.3)	40.0(39.2)	50.0(45.0)
9. ST with *T. koningii* @ 4 g/ kg seed	93.3(75.0)	20.0(26.6)	40.0(39.2)	46.7(43.1)	41.7(40.2)
10. T_9+ SA with *P. fluorescens charvae* @ 2.5 kg/ ha 20 DAS	93.3(75.0)	6.7(14.9)	26.7(31.1)	33.3(35.3)	58.3(49.8)
11. T_9+ SA with *P. fluorescens* 2 @ 2.5 kg/ha 20 DAS	93.3(75.0)	20.0(26.6)	33.3(35.3)	40.0(39.2)	50.0(45.0)
12.- T_5+ SA with *T. koningii* @ 2.5 kg/ha 20 DAS	93.3(75.0)	13.3(21.4)	33.3(35.3)	40.0(39.2)	50.0(45.0)
13. ST with *T. viride* 4 @ 4 g/ kg seed	93.3(75.0)	26.7(31.1)	33.3(35.3)	46.7(43.1)	41.7(40.2)
14. T_{13}+ SA with *P. fluorescens charvae* @ 2.5 kg/ ha 20 DAS	93.3(75.0)	20.0(26.1)	26.7(31.1	33.3(35.3)	58.3(49.8)
15. T_{13}+ SA with *P. fluorescens* 2 @ 2.5 kg/ ha 20 DAS	93.3(75.0)	26.7(31.1)	33.3(35.3)	40.0(39.2)	50.0(45.0)
16. T_{13}+ SA with *T. viride* 4 @ 2.5 kg/ ha 20 DAS	93.3(75.0)	20.0(26.6)	33.3(35.3)	40.0(39.2)	50.0(45.0)
17. SA with *L. edodes* @ 2.5 kg/ha at seed sowing	86.7(68.6)	267(31.1)	40.0(39.3)	66.7(54.7)	16.7(23.7)
18. T_{17}+ SA with *L. edodes* @ 2.5 kg/ ha 20 DAS	86.7(68.6)	20.0(26.6)	33.3(35.3)	66.7(54.7)	16.7(23.7)
19. T_1+ SA with *L. edodes* @ 2.5 kg/ ha 20 DAS	93.3(75.0)	13.3(21.4)	20.0(26.6)	53.3(46.9)	33.3(35.3)
20. T_5+ SA with *L. edodes* @ 2.5 kg/ha 20 DAS	93.3(75.0)	13.3(21.4)	26.7(31.1)	53.3(46.9)	33.3(35.3)

Treatment	Germination (%)*	Root-rot incidence (%)* DAS			Disease reduction over control at 75 DAS (%)
		45	60	75	
21. T_{19} + SA with *T. viride* @ 2.5 kg/ha 20 DAS	93.3(75.0)	13.3(21.4)	33.3(35.3)	53.3(46.9)	33.3(35.3)
22. T_{20} + SA with *T. viride* @ 2.5 kg/ha 20 DAS	93.3(75.0)	13.3(21.4)	26.7(31.1)	53.3(46.9)	33.3(35.3)
23. T_{21} + SA with *P. fluorescens charvae* @ 2.5 kg/ ha 20 DAS	86.7(68.6)	20.0(26.6)	40.0(39.2)	60.0(35.3)	25.0(30.0)
24. T_{22} + SA with *P. fluorescens* 2 @ 2.5 kg/ ha 20 DAS	86.7(68.6)	26.7(31.1)	40.0(39.2)	60.0(35.3)	25.0(30.0)
25. T_{23} + SA with *T. koningii* @ 2.5 kg/ ha 20 DAS	86.7(68.6)	26.7 31.1)	33.3(35.3)	60.0(35.3)	25.0(30.0)
26. T_{24} + SA with *T. viride* 4 @ 2.5 kg/ ha 20 DAS	86.7(68.6)	20.0(26.6)	40.0(39.2)	60.0(50.8)	25.0(30.0)
27. ST with carbendazim @ 2 g/kg seed	93.3(75.0)	26.7(31.1)	33.3(35.2)	40.0(39.2)	50.0(45.0)
28. T_{25} + SD with 0.1% carbendazim 20 DAS	100(89.7)	6.7(14.9)	20.0(26.6)	26.7(31.1)	66.7(54.7)
29. Control (With pathogen)	73.3(56.9)	46.7(43.1)	66.7(54.7)	80.0(63.4)	-
CD (P=0.05) : Root - rot incidence Treatments Interaction			0.86 0.27 0.47		

Figures in the parentheses are arcsine- transformed values; ST- Seed treatment; SA- Soil application; SD- Soil drench; DAS- Days after sowing

control and it was followed by *P. fluorescens* 2 which recorded 42.78 per cent growth inhibition. (Table 3). *P. fluorescens charvae* recorded the least mycelial growth of *M. phaseolina* (38.5 mm) at 3 DAI besides the sclerotial production was reduced to 67.5 numbers. Among the *Bacillus* isolates, *B. subtilis* 2 recorded least antagonistic effect of 26.22 per cent over the control and the number of sclerotia produced was increased to the tune of 99.25%. Among the six isolates of *Trichoderma* spp. tested against *M. phaseolina*, *T. koningii* inhibited the mycelial growth to the extent of 75.84 per cent and the number of sclerotia produced by *M. phaseolina* was also reduced to 43.57/ 9 mm disc (Table 4).

Among the various treatments under pot-culture condition on root-rot incidence, seed treatment with *P. fluorescens charvae* @ 10 g kg^{-1} of seed plus soil application @ 2.5kg ha^{-1} 20 DAS and seed treatment with carbendazim @ 2g kg^{-1} of seed plus soil drenching @ 0.1 per cent 20 DAS registered the least root-rot incidence of 26.66 per cent which accounted for 66.67 per cent disease reduction in 75 DAS.

It was followed by seed treatment with *P. fluorescens. charvae* @ 10g kg^{-1} of seed plus soil application with *T. koningii* @ 2.5 kg ha^{-1} (20 DAS), seed treatment with *T. koningii* @ 4 g kg^{-1} plus soil application with *P. fluorescens charvae* @ 2.5 kg ha^{-1} (20 DAS) and seed treatment with *T. viride* @ 4 g kg^{-1} plus soil application with *P. fluorescens charvae* @ 2.5 kg ha^{-1} (20 DAS) recorded the seed germination of 93.34; 93.34 and 93.34 per cent, respectively and these three treatments registered the minimum root-rot incidence (33.33%) only 75 DAS. This accounted for 58.34 per cent disease reduction as compared to control (Table 5).

In greenhouse study *P. fluorescens* and *T. koningii* were applied as talc powder (Vidhyasekaran and Muthamilan, 1995; 1999). Seed treatment of *T. koningii* @ 4 g kg^{-1} of seed plus soil application @ 2.5 kg ha^{-1} was recorded as best treatment in the pot-culture studies. The key of the competitive success of *Trichoderma* spp. is the ability of the fungus to tolerate the antagonistic activities of competing organisms in soil, leading to the extreme rapid growth and abundant production of spores, selective enzymes and antibiotics. As a result of seed treatment with talc-based formulation of *T. viride*, the plants recorded improved growth and higher population of *T. viride* throughout the growing period in various crop plants (Jeyarajan and Ramakrishnan, 1995). In the present study, biocontrol agents, like *P. fluroscens* and *T. koningii* were found to be effective in controlling root-rot incidence of cowpea. *P. fluorescens* and *T. koningii* reduced the mycelial growth of *M. phaseolina* as well as its sclerotial production. Hence, *T. koningii* was comparatively more effective than *P. fluorescens*.

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