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BIOLOGICAL AND CHEMICAL MANAGEMENT OF *PHYTOPHTHORA* ROOT ROT /COLLAR ROT IN CITRUS NURSERY

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ABSTRACT
Citrus jambhiri Lush is the most widely used root stock for most of citrus scion in India. Soil samples were collected from nurseries of Vidarbha region in India. Almost all samples were found associated with *P. parasitica* (28-46 cfu/g soil) when tested on PARPH medium. Rangpur lime was found tolerant root stock whereas, *Citrus jambhiri* was found susceptible to the disease. *In vitro* antagonism showed that *P. parasitica* significantly inhibited by *T. harzianum* and *T. virens* (84.96%). However, intensity of antagonism was different as per medium. There was a continuous reduction in pathogen population from 41 to 8 propagules/g soil with reduction in root rot /collar rot in *Citurs jambhiri*. All Thirty seven native isolates of *Pseudomonas* spp. were found positive for production of IAA, HCN and Siderophore. Pf XXVI (16.80%) and Pf IV (24.10%) were found effective to manage the disease in addition to increased growth response under glass house condition. Among chemicals, seed treatment with Metalaxyl @2.5 g/ Kg seed *f.b*. spraying of metalaxyl at 45 and 90 days after emergence was effective to manage the disease (8.56%).

INTRODUCTION

Phytophthora spp. are the causal agents of several serious diseases of citrus in India. *Phytophthora parasitica, P. citrophthora and P. palmivora* have been mostly involved in causing damping off, collar rot and root rot in citrus (Naqvi, 1988; Bowman *et al*., 2007; Shekari *et al.,* 2012). However, *Phytophthora parasitica* and *P. palmivora* are the most prevalent species in citrus orchards of Vidarbha region (Das *et al*., 2011). It remains a threat and a persistent problem wherever, citrus is grown that can result in substantial tree loss particularly trees on susceptible rootstock Whiteside (1974). Supply of poor quality sampling may result in foot rot, root rot or gummosis in orchard. These are the most important soil borne diseases of citrus causing mortality of newly planted trees and a slow decline and yield loss of mature trees (Graham and Menge, 1999). Epidemics of *Phytophthora* on heavy black cotton soils play an important role in citrus root stock failure. Survey have been undertaken to see the association of *Phytophthora* spp. in Vidarbha region where nearly 80L grafts are being raised for sale every year. Management of *Phytophthora* diseases includes the use of tolerant rootstocks and fungicides, but biological controls are not commercially available. Applications of the systemic fungicides metalaxyl and fosetyl-Al have been shown to increase fibrous root weight of citrus rootstocks and to reduce propagules of *P. parasitica* (Sandler *et al.,* 1989). However, fungicides may not always be economically justifiable (Graham and Menge, 1999). Therefore, applications are not recommended unless populations of *P. nicotianae* exceed a threshold of 10 to 15 propagules/cm^3 of soil (Graham and Menge, 1999; Sandler *et al*., 1989). Several potential biological agents have been investigated for the control of *Phytophthora* root rot of citrus (Armarkar, 2011). The ability of introduced antagonists to establish and colonize the rhizosphere soil is an important factor in successful biological control (Graham, 2004; Lewis and Papavizas, 1984). Studies involving control of *Phytophthora* root rot of citrus confirm the limitations of introduced biocontrol agents (Nemec *et al.,* 1996; Steddom *et al*., 2002). Weekly applications of *Pseudomonas putida* through the irrigation system were necessary to sustain the antagonist in the rhizosphere (Steddom *et al*., 2002). *Pseudomonas fluorescens* also proved to be effective against *Phytophthora in* citrus sp. (Gade and Armarkar, 2011). However, integration of *P. fluorescens* with metalaxyl and fosetyl- Al proved most effective in management of root rot in citrus (Koche, 2011). In the present study, objective was to identify resistant root stocks to *Phytophthora* spp has been identified and the bio-control agents have been evaluated to manage the disease when susceptible but popular root stock (*Citrus jambhiri*) is being used.

MATERIALS AND METHODS

Field nursery samples were taken from primary nursery of the citrus up to transplantation of seedling in secondary nursery beds. The number of samples collected varied with the size of nursery. At least 10 soil cores were taken from root zone from several rows of the field nursery. The samples were placed in plastic bags to maintain soil moisture, transported to laboratory, and assayed as described by Timmer *et al*. (1988).10g soil

from each sample was diluted in 90mL water having 0.25 % agar. One mL aliquot was spread on each of 10 plates of PARPH selective medium (Kannwischer and Mitchell, 1978). The plates were incubated at 28ºC for 2-3 days and no. of colonies of *Phytophthora* was counted. Soil in the second core was flooded with water, baited with pieces of citrus leaves, and placed in the incubator for 48h (Grimm and Alexander, 1973). The leaves were transferred to petridishes and examined for the presence of papillate sporangia.

Screening of root stocks

Seeds of rough lemon (*Citrus jambhiri*), cleopatra mandarin (*C. reticulata* Blanco) and Rangpur lime (*Citrus limonia*) were sown in pots. Pure culture of *Phytophthora parasitica* was maintained on V-8 juice agar by serial transfer. Chlamydospores of each isolate were produced in V-8 juice broth by the method of Tsao (1971) for use as inoculum. The soil was mixed manually to produce an inoculum concentrate. To determine the propagule concentration, 1g samples of the inoculum concentrate were plated on a selective medium containing pimaricin, ampicillin, rifampcin, pentac hloronitrobenzene and hymexazol (PARPH) (Timmer *et al*., 1988). The inoculum concentrate of each isolate was added to autoclaved fine sand to provide a density of 31-33 propagules/g of soil. Non inoculated pots were treated as control. For all treatments, 10 single seedlings in each replication and seven replications per treatment were used for each of the rootstock and placed in a completely random design in the greenhouse. Each pot was watered to runoff twice a day. Greenhouse conditions ranged from 25 to 35ºC and 60 to 100% relative humidity. Seedlings were evaluated 4 weeks after emergence. Seedlings were fully removed from the soil to avoid breakage of roots, and roots were washed free of the adhering soil. Root tips were examined and the percentage of root rot was calculated. Observations on growth parameters were also recorded. Data for the three rootstocks were analyzed in randomized block design.

Growth inhibition test

Purified cultures were maintained on corn meal agar. A 5 mm disc of *Phytophthora* culture was placed at one side of the previously plated 90mm diameter with 20mL of different media (V-8 juice, CMA, PDA and 2% Agar) and 5 mm disc of *Trichoderma* (individual species) was placed at opposite side of *Phytophthora* disc. These Petri plates were incubated at 25ºC. Three plates were used for each replication and three replications were used for each treatment.

Characterization of *Pseudomonas*

Siderophore production

Production of siderophore by *P. flourescens* was assessed by Plate assay method as described by Schwyn and Neilands (1987).

Hydrocyanic acid production

HCN production was tested by the method of Castic and Castric (1983).

Indole acetic acid production

Indole Acetic acid production was tested according to (Gorden and Webber, 1951).

Gelatin liquefaction

The test indicated utilization of protein and production of proteolytic enzymes by bacterium and to differentiate *Pseudomonas fluorescens and P.putida*

Oxidase test

Take an inoculating loop or toothpick. Then touch and spread a well isolated colony on an oxidase disk (Disk contains N, N-dimethyl-p-phenylenediamine oxalate and α-naphthpol).The reaction was observed within 2 minutes at 25-30ºC. Deep purple blue indicate positive reaction.

Arginine test

For Arginine test media was made according to the method of Fay and Berry (1972). Purple colour indicates positive reaction and yellow colour or no colour change indicates negative reaction.

Nitrate reduction

The nitrate broth medium will be inoculated with the bacteria and inoculated at 37ºC for 48h or until the next period. To each tube 1mL of sulphanillic acid and napthylamineacetate is to be added. Reduction of nitrite to nitrate is indicated by the production of distinct red colouration. Comparison was made with the blank. On the basis of characteristics and by dual culture test antifungal activity of *P. fluorescens* isolates were identified and selected on the basis of their inhibition activity for further study.

Use of antagonist and fungicides for disease management

Citrus jambhiri seeds which is a susceptible root stock to *Phytophthora* bacterized with *P. fluorescens* isolates (Pf-I, Pf-IV, Pf-XXVI) @10g/kg seed and for *Trichoderma* spp. seeds were treated @4g/kg seed. For fungicides, seeds were treated with the formulation of metalaxyl @2.5 g/kg seed and 2g/L water for spraying similar concentrations were used for fosetyl - AL. Similar procedure was followed as described in screening of root stock for development of sickness in soil.

RESULTS

Survey of Amravati and Nagpur district citrus nurseries was done where nearly 80 lakhs grafts of citrus are being produced every year. All most all the samples in Nagpur and Amravati district were tested positive to *Phytophthora*. Propagule densities of soil was in the range of 28.00-38.67 cfu/g soil in Amravati and 29.11 to 46.33 cfu/g soil in Nagpur district. However, *Phytophthora* was not detected in each one of the field nursery of Amravati and Nagpur district. The leaf baiting technique detected more positive samples as compared to selective medium when the propagule density was high. It was failed to produce results when the propagule density was low (Table 1).

Screening of root stock

All three rootstock screened in this experiment were polyembryonic and produce high proportion of seedlings. Seedling emergence began at nearly 21 DAS. Post emergence seedling root rot were noted in all three rootstocks. In the first two weeks after emergence of the seedlings death was preceded by severe leaf yellowing and necrosis at collar region of the seedlings (Table 2). Per cent root rot was significantly

Table1: Occurrence of *Phytophthora* among citrus nurseries in Amravati and Nagpur District of Vidarbha region

Locations District Amravati	No. of samples	Selective Media No of positive samples	Propagules /g soil	Positive in leaf baiting technique
1.	09	08	28.00	06
2.	13	13	38.67	13
3.	06	04	31.50	05
4.	10	08	34.50	09
5.	04	04	29.67	03
6.	15	11	34.67	13
7.	08	06	32.83	06
8.	11	07	36.29	10
9.	06	05	28.67	02
10.	05	04	35.50	05
11.	09	07	34.86	08
12.	08	06	34.67	07
13.	01	00	0.00	00
14.	06	06	35.67	06
15.	10	07	35.29	09
Nagpur				
1.	08	08	35.33	08
2.	06	05	30.00	01
3.	04	02	34.67	03
4.	03	00	0.00	02
5.	05	04	29.11	02
6.	04	04	33.11	04
7.	04	02	36.50	03
8.	06	04	29.67	02
9.	03	01	46.33	02
10.	03	03	30.67	01

Table 2: Response of citrus root stock to *Phytophthora parasitica*

Rootstock	Root Fresh wt (g)	Length (cm)	Shoot Fresh wt (g)	Length (cm)	Root rot(%)
Rangapur Lime	2.07	41.60	7.15	30.93	24.67 (29.76)
Rough lemon	2.19	38.57	6.93	36.60	43.33(41.16)
Cleopatra mandarin	2.56	46.20	8.25	37.77	31.33(33.99)
CD (P=0.01)	0.10	1.03	0.96	2.74	1.44

Values in parenthesis are arc sin means

higher in *Citrus jambhiri* (43.33 %) as compared to other two root stock under screening. Root rot per cent was significantly low in rangpur lime (24.67%). However, significant increase in root fresh wt (2.56g), root length (46.20cm), shoot fresh wt (8.25g) and shoot length (37.77cm) was found in Cleopatra mandarin.

Observations on average colony diameter and per cent growth inhibition were recorded. All *Trichoderma* spp. under the test showed their efficacy to check the mycelial growth of the pathogen on the entire medium under test. The data presented in Table 3. Indicate that all treatments were effective to inhibit the mycelial growth of *P. parasitica* except control. *T. virens* was found significantly superior to inhibit the mycelial growth of *P. parasitica* on the entire medium (V-8 Juice 16.70mm, CMA 17.40mm, PDA 10.30mm, 2% Agar 5.20mm) as compared to all other treatments.

Characteristics of *Fluorescent pseudomonads*

Out of 37 Pseudomonas species 19 were *P. fluorescens* 11 were *P. putida* and 7 were *P. aurigonosa* on the basis of biochemical test. The results of the test are shown in Table IV. All *Pseudomonads* were found positive to oxidase where, microdase disck were turned to deep purple blue; IAA production, where, development of cherry red colour was observed when Kovac's indole reagent was added; HCN production, where, change in colour from yellow to light brown, brown or reddish brown of filter paper soaked in 0.5 % picric acid in 1 % Na_2Co_3 was observed and siderophore production where orange colour zone after 48h. of incubation was observed around the colonies. However, *P. aeruginosa* was found negative to Arginine and positive to *P. fluorescens* and *P. putida* whereas *P. putida* was found negative to gelatin liquefaction and nitrate reduction and also *P. fluorescens* was found negative to nitrate reduction (Table 4).

Table 4: Characteristics of *P. fluorescens, P. putida* and *P. aeruginosa*

Characteristics	*P. fluorescens*	*P. putida*	*P. aeruginosa*
No.of Isolates	19	11	7
Oxidase	+ ve	+ ve	+ ve
Nitrate reduction	-ve	-ve	+ ve
Gelatin liquefaction	+ ve	-ve	+ ve
Arginine test	+ve	+ve	-ve
IAA	+ ve	+ ve	+ ve
HCN	+ve	+ ve	+ ve
Siderophore	+ ve	+ ve	+ ve

All *Trichoderma* spp and *P. fluorescens* significantly reduce root rot and stimulated the growth of the seedlings of *Citrus jambhiri*. *T. virens* was found effective to lower down propagule count of *Phytophthora* in the soil (8 cfu/g soil). Seed treatment with metalaxyl @ 2.5 g/kg seed *f.b.* spraying @ 0.2% at 45 and 90 DAE was found significantly superior to manage root rot per cent than all other treatments but at par with seed treatment with metalaxyl @ 2.5g/kg seed *f.b.* spraying of alliette @0.2% at 75 DAE. Amongst bioagents *P. fluorescens* XXVI (16.80%) was found effective to lower down the root rot incidence f.*b*. *T. virens* (17.50%). Root (2.92g) and shoot fresh wt. (8.51g) were found maximum in *P. f.* XXVI (Table 5).

DISCUSSION

Field nurseries were found contaminated with *Phytophthora*. It may be due to rising of nurseries on same piece of land and

Table 3: Assessment of Antagonism of *Trichoderma* spp. against *Phytophthora parasitica* on different medium

Antagonists	Mean colony diameter of *P.parasitica* (mm)				Per cent inhibition			
	V-8 juice	CMA	PDA	2% Agar	V-8 juice	CMA	PDA	2% Agar
Trichoderma virens	16.70	17.40	10.30	5.20	81.44	80.67	85.39	37.35
Trichoderma viride	17.30	18.00	10.60	6.10	80.78	80.00	84.96	26.51
Trichoderma harzianum	17.30	17.60	10.60	6.00	80.78	80.44	84.96	27.71
Trichoderma hamatum	18.00	18.60	13.00	6.50	80.00	79.33	81.56	21.69
Control	90.00	90.00	70.50	8.30	0.00	0.00	0.00	0.00
CD(P=0.01)	0.41	0.49	0.28	0.62	-	-	-	-

Table 5: Effect of bioagents and fungicides on occurrence of *Phytophthora* propagules /g soil, root and shoot fresh wt. and per cent root rot due to *P. parasitica*

Bioagents/ Fungicides	Cfu/g soil	Root rot(%)	Root fresh wt (g)	Shoot fresh wt.(g)
Trichoderma virens	08	17.50 (24.73)	2.72	7.92
Trichoderma viride	12	19.60 (26.26)	2.54	7.20
Trichoderma harzianum	11	26.40 (30.91)	2.65	7.25
Trichoderma hamatum	21	32.70 (34.87)	2.36	6.93
Pseudomonas fluorescens -I	22	29.20 (32.70)	2.33	6.10
Pseudomonas fluorescens- IV	16	24.10 (29.38)	2.87	8.34
Pseudomonas fluorescens XXVI	12	16.80 (24.19)	2.92	8.51
Seed treatment with metalaxyl @2.5g/kg Seed f.b. spraying @0.2% at 45 and 90 DAE	11	8.56 (17.01)	2.56	7.16
Seed treatment with metalaxyl @2.5g/kg seed f.b. spraying of Alliette @0.2% at 75 DAE	21	9.71 (18.14)	2.40	6.92
Control	41	44.20 (41.67)	1.38	3.95
CD(P = 0.01)	-	1.67	0.62	1.05

Values in parenthesis are arc sin means; DAE- Days after emergence

to the roadside to attract the customer and also there is no restriction to any person to enter in the nursery. Shekari *et al*. (2012) determined the soil population of each species and found that 82 and 86% of the orchards were infested respectively with *P.citrophthora* and *P. nicotianae* with average over 10 propagules/g soil. Ridings *et al.,* (1977) showed that even with strict sanitary practices, recontamination of disinfected areas occurred when it was present near to the nursery. The use of selective medium was as effective in the detection of *Phytophthora* as the leaf baiting technique. Therefore, selective medium would be useful for detection of *Phyotphthora* spp. where laboratory facilities are available (*Zitko et al.,* 1987). Three citrus rootstocks were screened to identify those that were highly resistant to root rot. Root rot incidence was observed low in Rangpur lime as compared to Rough lemon and Cleopatra mandarin. It suggests that Rangpur lime show potentiality as superior root stock because of their high tolerance to *Phytophthora* root rot (Armarkar, 2011). Cleopatra mandarin and sour orange are said to be highly resistant to infection by *Phytophthora* (Timmer *et al*., 1988). However, Cleopatra mandarin was recognized as susceptible to *Phyotphthora* (Anonymous, 1991). CMA and V-8Juice was found suitable medium for the growth of *Phytophthora*. Faster growth was observed on CMA, whereas, lowest growth rate was recorded in 2 % Agar (Naqvi, 2005). Meyer and Abdallah (1978) reported that *Pseudomonas* spp. are all members of the some intrageneric homology group. They include *P. aeruginosa*, *P. putida and P. fluorescens*. They are well known for production of broad spectrum antibiotics. It is proved to be a major mechanism involved in their biocontrol activity (O' Sullivan and O' Gara, 1992). HCN and siderophore produced by *Pseudomonas* spp. were also involved in their antifungal activity. Voisard *et al*., (1989) observed suppression of black rot of tobacco was due to the production of HCN by *P. fluorescens* and also HCN induced resistance in the host plant. In the present study, all selected antifungal *Pseudomonas* isolates were observed to produce HCN *in vitro*, which might have contributed for their biocontrol ability in addition to antibiotics (Gade and Armarkar, 2011). One of the proposed mechanisms of plant growth promotion by bacteria was production of IAA, cytokinin and Gibberellins (Glick, 1991). All four species of *Trichoderma* tested in the experiment provide significant disease control and enhanced plant growth (Jagtap *et al*., 2012). The reduction of root rot by *T*. spp. may be due to high antagonistic potential that includes antibiosis, parasitism and production of lytic enzymes (Singh *et al*., 2004). *Trichoderma virens* recorded minimum mean colony diameter on 2% agar and highest inhibition 85.39% of mycelial growth of *P. nicotianae* on PDA over untreated control followed by the bioagent *T. viride* and *T.harzianum*. *P. fluorescens* XXVI and IV were also found effective to manage the disease in addition to plant growth promotion activity. Yang *et al.,* (1994) reported that *Pseudomonas putida* 6909 and *Pseudomonas fluorescens* 09906 suppressed population of *Phytophthora parasitica* in the citrus rhizosphere, suggesting these bacteria may be useful in control of citrus root rot (Gade *et al*., 2008). Amongst fungicides, seed treatment with metalaxyl @2.5g/kg seed *f.b.* spraying @0.2% at 45 and 90 DAE was found effective. Naqvi (1993) conducted an experiment to determine effect of certain systemic and non systemic fungicide on soil population of *Phytophothora parasitica* and found that metalaxyl was more promising than fosetyl-AL because metalaxyl directly kills the pathogen *in vivo*. However, Graham and Timmer (2003) reported that metalaxyl and fosetyl AL are highly effective against *Phytophthora* spp.; they are often used routinely by nurserymen to suppress *Phytophthora* populations and reduced root rot damage in citrus nursery stock. Gade and Giri (2005) observed significant reduction in population of *Phytophthora* sp. (11.25 cfu/g soil) in beds priorly treated with solarization and then drenched with metalaxyl @ 0.2% alternate at bimonthly interval. Significant decrease in mortality was also recorded in same treatment (5.78%) with added benefit of increase in height and girth of *Citrus jambhiri* seedlings (Gade *et al*., 2008).Drenching of metalaxyl @ 0.2 per cent reduced mortality in citrus caused due to *Phytophthora* (13.9%) with added benefit of plant height (52.9cm) and girth (Gade *et al*., 2005).

From the survey of citrus nurseries it is observed that field nurseries must have an alternative which is found to be contaminated with *Phytophthora*. Root stocks study warranted the use of *Citrus jambhiri* which is found susceptible to *Phytophthora*. Production of antibiotics, IAA, HCN and siderophore by *P. fluorescens* and production metabolites by *Trichoderma* spp. will play major role in suppression of root rot and enhancement of plant growth in citrus nurseries.

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