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TESTING OF EFFICACY OF BIOAGENTS AND BOTANICALS AGAINST PHYTOPHTHORA ROOT ROT IN NAGPUR MANDRIN

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ABSTRACT

An experiment was conducted in which different combinations of botanicals *viz.*, Neem, Tulsi, Onion, Garlic, and Chrysanthemum and bioagents were used under *in vitro* and green house conditions for reducing the intensity of *Phytophthora* root rot. The bioagent *Trichoderma viride* gave highest growth inhibition (75.33%) of *Phytophthora parasitica* by dual culture method. In poisoned food technique complete inhibition of *Phytophthora parasitica* was recorded in Garlic (5%), whereas Tulsi found to be less effective to inhibit the growth of *P. parasitica* (54.44%). Under green house experiment combined application of *Trichoderma viride* @ 4g/kg + Garlic clove extract @ 5% reduced per cent root rot incidence (11.32%). Similar treatment showed maximum shoot and root length (4.5cm, 5.96cm) of *Citrus jambhiri*.

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INTRODUCTION

The Citrus is one of the most important group of fruit crops worldwide, belongs to the family Rutaceae comprising 140 genera and 1300 species distributed throughout the world. It is a long-lived perennial crop and is grown in more than 100 countries across the world (Saunt, 1990; Savita *et al.*, 2012). Citrus plants are prone to attack of more than 150 diseases and disorders caused by fungal, viral and few bacterial pathogens right from nursery level to bearing stage resulting in incalculable losses. *Phytophthora* spp. are the most destructive plant pathogens known to have a wide host range, severely affects citrus orchards and nurseries throughout the world (Erwin and Ribeiro, 1996; Das *et al.*,2013). The chemical treatments to control the pathogen have become recurred costly affairs. At present there is a need to develop and utilize the effective low cost, ecofriendly technologies in the crop production programme. The use of botanicals such as Neem, Tulsi, Onion, Chrysanthemum, Garlic and bioagents *Pseudomonas fluorescens* and *Trichoderma* has a good prospect in future as it can lead to a high cost benefit ratio. Keeping in view, the present investigation was undertaken to evaluate the botanicals and bioagents against *Phytophthora* root rot of Citrus.

MATERIALS AND METHODS

Collection of *Phytophthora* infected soil samples from major Citrus growing regions of Vidarbha

Phytophthora parasitica isolate was obtained from *Phytophthora* infected soil samples from major citrus growing regions of Vidharbha. The isolates were obtained from different places *viz.*, Akola, Buldhana, Amravati, Nagpur, Akot and Katol.

Isolation of *Phytophthora parasitica* from soil

For the isolation of *Phytophthora parasitica* PARPH-CMA selective medium was used (Jefferson *et al.*, 2000). Soil samples were collected from rhizosphere of citrus plants in orchards and nurseries from Vidarbha region. Seven soil cores were taken from root zone. The samples were placed in plastic bags to maintain soil moisture, transported to laboratory and assessed for population count of *Phytophthora parasitica* (Timmer *et al.*, 1988). Soil (10g) from each sample was diluted in 90 ml water having 0.25% agar. One ml aliquot was spread on each of 10 plates of PARPH-CMA selective medium. The plates were incubated at 28°C for 2-3 days and number 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 48hr (Grimm and Alexander, 1973). The leaves were transferred to Petri dishes and examined for the presence of papillate sporangia. Subculture of *Phytophthora* from infected leaf pieces was easily accomplished by submerging infected leaf pieces in PARPH-CMA medium and transferring hyphal tips as they grew in the medium. As soon as the growth of fungus was observed, small portion was transferred to the slants of the medium in test tube. Fungal cultures were observed under microscope for identification and the fungus was identified as *Phytophthora* based on the published literature.

Isolation of *Pseudomonas fluorescens* and *Trichoderma viride*

Soil samples from rhizosphere of citrus orchards and nurseries were collected from different locations of citrus growing areas of Vidarbha region. King's B medium was used for isolation of *P. fluorescens* (Kings *et al.,* 1954). One ml of soil suspension from aliquot dilutions 10^5 to 10^8 was aseptically added to sterile Petri plates containing twenty ml of sterile medium and incubated at 28 ± 2°C for 48 h. After incubation, well separated individual colonies with yellow green pigments were marked and detected by viewing under UV light. After 48 hr. Individual colonies were picked up with sterile loop and transferred to fresh King's B slants and the pure cultures so obtained were stored in a refrigerator at 4°C for further use. For isolation of *Trichoderma* soil sample was serially diluted and plated on *Trichoderma* selective media and incubated for 7 to 10 days. Sub culturing with actively growing colony of *Trichoderma* sp. was done on selected and plated on PDA medium.

Preparation of 5% concentration of water extract of selected plant part

Each sample of 100 g of plant part of Neem, Tulsi, Onion, Garlic, and Chrysanthemum was separately ground and homogenized in mechanical grinder with equal quantity of sterile distilled water (1:1, w:v). The homogenate obtained was then strained though double layered muslin cloth and the filtrate was collected and filtered through Whatman No. 1 filter paper using volumetric flasks (50 ml capacity). The clear leaf extracts obtained from the stock solution was 100%. An appropriate quantity of each leaf extract was incorporated separately in the molten and cooled potato dextrose agar (PDA) medium in conical flask (250 ml capacity) to get desired concentrations (5%) of each extract and autoclaved at 15 lbs pressure for 15 to 20 min. Plant extracts amended PDA was then poured (15 to 20 ml/plates) in sterilized Petri plates (90 mm diameter) under aseptic conditions. (Jagtap *et al.,* 2012).

***In vitro* evaluation of botanicals against *Phytophthora parasitica* poisoned food technique**

For evaluation of different botanicals by poisoned food technique, leaves of Neem, Tulsi, clove of Garlic, flowers of Chrysanthemum, red Onion bulbs were collected from the field of College of Agriculture, Akola. These botanicals were crushed with mortal and pistal. Extract of 5 ml Neem, Tulsi, Onion, Chrysanthemum and Garlic was taken and poured in 95 ml luke warm PDA in 250 ml conical flask. These flasks were plugged with non absorbent cotton and sterilized in autoclave at 15 lbs pressure psi for 15 min. After sterilization, PDA of each botanical was poured in sterilized Petri plates. For each botanical 3 Petri plates were taken. After cooling, 5 mm disc of *Phytophthora* sp. was inoculated in each Petri plate. Observations on colony growth were taken by measuring the vertical and horizontal diameter of the colony. One set of 3 Petri plates without botanical extract was maintained as control for comparison (Jagtap *et al.,* 2012).

Efficacy of bioagent against *Phytophthora parasitica* by dual culture technique

The antagonistic potential of *T. viride* and *Pseudomonas fluorescens* was assessed against *Phytophthora parasitica* by dual culture technique on PDA medium.

Green house experiment

Experiment was conducted against the target pathogen (*Phytophthora parasitica*) under green house conditions by using seeds of *Citrus jambhiri*. The trial was conducted in randomized block design with three replications. Different treatments used under the experiment *viz.,* T_1 - Seed treatment with *T. viride* @ 4g/kg of seed + Neem leaf extract @ 5%, T_2 - Seed treatment with *P. fluorescens* @ 10g/kg of seed + Neem leaf extract @ 5%, T_3 - Seed treatment with *T. viride* @ 4g/kg of seed + Chrysanthemum flower extract @ 5%, T_4 - Seed treatment with *P. fluorescens* @ 10g/kg of seed + Chrysanthemum flower extract @ 5%, T_5 - Seed treatment with *T. viride* @ 4g/kg of seed + Tulsi leaf extract @ 5%, T_6 - Seed treatment with *P. fluorescens* @ 10g/kg of seed + Tulsi leaf extract @ 5%, T_7 - Seed treatment with *T. viride* @4g/kg of seed + Onion bulb extract @ 5%, T_8 -Seed treatment with *P. fluorescens* @10g/kg of seed + Onion bulb extract @5%, T_9 - Seed treatment with *T. viride* 4g/kg of seed + Garlic clove extract @ 5% , T_{10} -Seed treatment with *P. fluorescens* @ 10g/kg of seed + Garlic Clove extract @ 5%, T_{11} - Untreated Control.

RESULTS AND DISCUSSION

Collection of soil sample and Isolation of *Phytophthora* spp.:

The soil samples from rhizosphere of citrus were collected from different locations of citrus growing areas. For isolation of *Phytophthora,* leaf bait technique, soil spreading and tissue isolation methods were followed. Amongst all the isolation methods tested, leaf baiting technique was found to be most effective. *Phytophthora* was isolated on PARPH medium (Jefferson *et al.,* 2000). Zitko *et al.* (1987) detected the *Phytophthora* in 8 to 15 field nurseries by leaf baiting technique with propagules density of 1 to 59 propagules/ cm^3 of soil. Gade and Armarkar (2011) also observed *Phytophthora parasitica* on PARPH medium. Gade (2012) obtained colonies of *Phytophthora* from soil samples collected from citrus nursery.

Efficacy of 5% botanicals against *Phytophthora parasitica* by poisoned food technique

Five botanicals *viz.*, Neem, Tulsi, Onion, Chrysanthemum and Garlic were evaluated @ 5% concentration *in vitro* against *Phytophthora parasitica* by applying poisoned food technique. The data presented in Table 1 indicated that, all the botanicals were inhibitory and caused significant inhibition of mycelial growth of the *Phytophthora parasitia* over untreated control. Among five botanicals, 100% inhibition of mycelial growth of *Phytophthora parasitica* was observed in Garlic which contains diallyl disulphide, diallyl sulphide, diallyl trisulphide, allyl propyl disulphide, allyl alcohol, dimethyl trisulphide, allyl methyl trisulphide and allycin (diallyl trisulphonate) in its aromatic oils and these compounds have antimicrobial effects (Akgul, 1993; Ashurst, 1995; Schwartz and Mohan,1995). This may be the reason to get cent per cent reduction in growth of *P. parasitica*. This was followed by Neem (69.63%) which contains Azadirachtin and gedunin which possess antifungal properties (Sadri *et al.,* 1983) which was followed by Onion (65.18%), Chrysanthemum (62.96%) and Tulsi (54.44%). The

Table 1: *In vitro* efficacy of botanicals against *Phytophthora parasitica*

Bioagents	*Phytophthora parasitica* Colony diameter (mm) 8 DAI	Per cent inhibition 8 DAI
Trichoderma viride	22.2	75.33
Pseudomonas fluorescens	26.8	70.22
Control	90.00	0.00
CD (p=0.01)	2.46	

Table 2: *In vitro* efficacy of bioagents against *Phytophthora parasitica*.

Common name	Scientific name	Concentration	Colony diameter (mm)	Per cent Inhibition
Neem leaf extract	*Azadiracta indica*	5%	27.33	69.63
Tulsi leaf extract	*Ocimum sanctum*	5%	41.00	54.44
Chrysanthemum flower extract	*Chrysanthemum indicum*	5%	33.33	62.96
Onion bulb extract	*Allium cepa*	5%	31.33	65.18
Garlic clove extract	*Allium sativum*	5%	0.00	100
Control			90.00	00
CD (P=0.01)			4.88	

Table 3: Combined effect of botanicals and bioagents on per cent root rot in *Citrus jambhiri*

Treatments	Per cent disease Incidence on *Citrus jambhiri* 30 DAE Root rot
T_1 - ST with *Trichoderma viride* @ 4g/kg seed + Neem leaf extract @ 5%	11.79(22.04)**
T_2 – ST with *Pseudomonas fluorescens* @ 10g/kg seed+ Neem leaf extract @ 5%	14.58(24.47)
T_3 - ST with *Trichoderma viride* @ 4g/kg seed + Chrysanthemum flower extract @ 5%	16.66(25.96)
T_4 – ST with *Pseudomonas fluorescens* @ 10g/kg seed + Chrysanthemum flower extract @ 5%	19.04(27.14)
T_5 – ST with *Trichoderma viride* @ 4g/kg seed + Tulsi leaf extract @ 5%	22.52(29.31)
T_6 – ST with *Pseudomonas fluorescens* 10g/kg seed+ Tulsi leaf extract @ 5%	25.75(31.87)
T_7 – ST with *Trichoderma viride* 4g/kg seed + Onion bulb extract @ 5%	15.55(23.46)
T_8 – ST with *Pseudomonas fluorescens* 10g/kg seed + Onion bulb extract @ 5%	15.87(23.46)
T_9 – ST with *Trichoderma viride* 4g/kg seed + Garlic clove extract @5%	11.32(22.04)
T_{10} – ST with *Pseudomonas fluorescens* 10g/kg seed + Garlic clove extract @ 5%	12.53(22.60)
T_{11} – Untreated Control	49.99(42.67)
CD (P= 0.05)	4.64

highest reduction in the mycelial growth of *P. Parasitica* was recorded by Garlic (0.00 mm) which was followed by Neem (27.33 mm), Onion (31.33 mm), Chrysanthemum (33.33mm) while, Tulsi found less effective which recorded minimum inhibition of mycelial growth of *P. parasitica* (41.00 mm) respectively. The maximum mycelial growth of *P. parasitica* was noted in control plate (90.00 mm). The present results are more or less similar with the findings of Rashid *et al.* (2004) who noted that neem leaf diffuses and neem leaf powder completely inhibitory to *Phytophthora infestans*. Kassa *et al.* (2006) indicated that the highest concentration 8% of crude garlic extract showed significantly highest (99.5%) mycelial growth which was comparable to the 0.1% fungicide amended treatment (100%). Jagtap *et al.* (2012a) also reported the highest mean inhibition of mycelia growth of *P. nicotianae* (47.26%) by garlic extract which was followed by Neem (38.65%), Onion (32.38%), Ginger (27.26%), and Tulsi (22.81%). The minimum mean inhibition of mycelia growth of *P. nicotianae* was found in turmeric extract (20.27%), which was found least effective.

Efficacy of bioagents against *Phytophthora parasitica* by dual culture technique

Efficacy of *Trichoderma viride* and *Pseudomonas fluorescens* were tested against *Phytophthora parasitica* on PDA medium by dual culture technique. Observations on average colony diameter of *Phytophthora parasitica* were recorded eight days after incubation. Data presented in Table 2 revealed that both the bioagents tested were found effective in the inhibition of mycelial growth of *Phytophthora parasitica*. There was highest reduction in mycelial growth of *Phytophthora parasitica* by *Trichoderma viride* (22.2 mm) and minimum reduction of mycelial growth of *Phytophthora parasitica* was observed by *Pseudomonas fluorescens* (26.8 mm). The maximum mycelial growth of *Phytophthora parasitica* was noted in control (90.00 mm). The maximum per cent growth inhibition of *Phytophthora parasitica* was observed in treatment of *Trichoderma viride* (75.33%) and minimum per cent inhibition of mycelial growth of *Phytophthora parasitica* was observed in *Pseudomonas fluorescens* (70.22%) after inoculation. The above findings are in conformity with Elad *et al.* (1982) who reported that *Trichoderma* spp. and bacterial bioagents produce mycolytic enzymes which play an important role in the degradation of target pathogens. Paul *et al.* (2005) found that strains of *Pseudomonas fluorescens* caused cytoplasmic coagulation in the mycelium of *Phytophthora* sp. when they were cultured together and hyphal colonization. Singh and Islam (2010) found that all three isolates of *Trichoderma harzianum* and one of *T. viride* showed considerable antagonistic potential for the biocontrol of *P. nicotianae* isolate,

Table 4: Combined effect of botanicals and bioagents on shoot length and root length in *Citrus jambhiri*.

Treatments	*Citrus jambhiri*			
	Shoot length(cm) 30 DAE	Shoot length (cm) 60 DAE	Root length (cm) 30 DAE	Root length (cm) 60 DAE
T_1 - ST with *Trichoderma viride* @ 4g/kg seed + Neem leaf extract @ 5%	3.40	4.43	4.70	5.83
T_2 – ST with *Pseudomonas fluorescens* @ 10g/kg seed+ Neem leaf extract @ 5%	3.33	4.26	4.60	5.76
T_3 - ST with *Trichoderma viride* @ 4g/kg seed + Chrysanthemum flower extract @ 5%	3.20	4.10	4.46	5.56
T_4 – ST with *Pseudomonas fluorescens* @ 10g/kg seed + Chrysanthemum flower extract @ 5%	3.16	3.90	4.36	5.46
T_5 – ST with *Trichoderma viride* @ 4g/kg seed + Tulsi leaf extract @ 5%	3.13	3.83	4.23	5.30
T_6 – ST with *Pseudomonas fluorescens* 10g/kg seed+ Tulsi leaf extract @ 5%	3.03	3.73	4.13	5.26
T_7 – ST with *Trichoderma viride* 4g/kg seed + Onion bulb extract @ 5%	3.26	4.23	4.56	5.70
T_8 – ST with *Pseudomonas fluorescens* 10g/kg seed + Onion bulb extract @ 5%	3.26	4.13	4.50	5.66
T_9 – ST with *Trichoderma viride* 4g/kg seed + Garlic clove extract @5%	3.46	4.50	4.76	5.96
T_{10} – ST with *Pseudomonas fluorescens* 10g/kg seed + Garlic clove extract @ 5%	3.36	4.30	4.63	5.80
T_{11} – Untreated Control	2.53	3.10	3.70	5.00
CD (P= 0.05)	0.13	0.20	0.16	0.18

ST- Seed treatment

in which *T. harzianum* (0034H) was found highly inhibitory to *P. nicotianae* in dual culture followed by *T. harzianum* (0034M) and *T. harzianum* (0034W). *T. viride* (0034S) was least effective to inhibit the mycelial growth of *P. nicotianae*. Jagtap *et al.* (2012) reported that *Trichoderma harzianum* (87.85%) caused maximum per cent inhibition of mycelial growth of *Phytophthora nicotianae* which was followed by *Trichoderma viride* (84.36%), *T. koningii* (77.76%), *T. hamatum* (75.40%), *Gliocladium virens* (71.69%) and *Pseudomonas fluorescens* (70.31%), respectively.

Green house Experiment

Experiment was conducted to see the effect of combination of botanicals and bioagents on root rot and shoot and root length of *citrus jambhiri*.

The data on combined effect of botanical and bioagents on per cent root rot in *citrus jambhiri* presented in table 3 revealed that all treatments reduced the intensity of root rot. The combination of seed treatment with *Trichoderma viride* 4g/kg seed + Garlic clove extract @ 5% i.e. T_9 was superior among all treatments which recorded lowest root rot at (11.32%) 30 DAE which was at par with treatment T_1 i.e. *Trichoderma viride* @ 4g/kg seed + Neem leaf extract @ 5% where 11.79% incidence in root rot was recorded at 30 DAE, this was followed by T_2 i.e. *Pseudomonas fluorescens* @ 10g/kg seed+ Neem leaf extract @ 5% in which 14.58% root rot was observed at 30 DAE. However, T_6 i.e. *Pseudomonas fluorescens* 10g/kg seed+ Tulsi leaf extract @ 5% was found less effective among all treatments which recorded highest (25.75%) root rot at 30 DAE.

The results found in present investigation corroborates with the findings of Yang *et al.* (1994) who 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. Kassa *et al.* (2006) reported that Garlic extract at 2% had significant influence and strongly inhibited the infection of *P. infestans* when it was applied 1 day earlier at the same time of inoculation and showed 92% and 100% inhibition effects in terms of lesion number and lesion size, respectively. Seed treatment with Pf-IV isolate of *Pseudomonas fluorescens* @10g/kg seed and soil application was found effective to manage damping off and root rot in acid lime (Gade *et al.* 2008). Gade (2012) reported that Pf XXVI (16.80%) and Pf IV (24.10%) strains of *Pseudomonas fluorescens* were effective to manage the disease in addition to increased growth response under glass house condition. The decrease in the intensity of root rot and gummosis in Nagpur mandarin was also reported by Gade and Koche (2012).

The data presented in Table 4 showed that highest shoot length (3.46cm) was observed at 30 DAE and 4.5cm at 60 DAE, respectively which was recorded in T_9 *i.e. Trichoderma viride* 4g/kg seed + Garlic clove extract @ 5%.while, similar treatment gave highest root length (4.76cm) at 30 DAE and at 60 DAE (5.96cm) which was at par with T_1 i.e. *Trichoderma viride* @ 4g/kg seed + Neem leaf extract @ 5% which recorded 3.4cm shoot length at 30 DAE and 4.43cm at 60 DAE. While, similar treatment gave root length 4.7cm at 30 DAE and 5.83cm at 60 DAE. Whereas, T_6 i.e. *Pseudomonas fluorescens* 10g/kg seed+ Tulsi leaf extract @ 5% was found less effective which recorded minimum shoot length at 30 DAE (3.03cm) and at 60 DAE (3.73cm) and minimum root length at 30 DAE(4.13cm) and at 60 DAE (5.26cm).

The results are in accordance with the findings of Mahesh (2007) who observed the maximum seedling emergence (46.00), shoot length (16.25cm) and root length (10.45cm) with seed treatment with *Pseudomonas fluorescens* (PfIV) and soil drenching of PfIV at 15 and 30 DAS. Armarkar (2011) observed that seed treatment with *Pseudomonas fluorescens* (Pf_2) @ 10g/kg seed showed maximum shoot length and root length at all the periods *i.e.* (11.32cm) and (7.80 cm), respectively followed by seed treatment with Pf_{26} @ 10g/kg seed at 90 DAE as against control which showed minimum shoot length (5.91cm) and root length (2.20 cm) at 90 DAE. This may be because of growth promoting activity of *P. fluorescens* which produce IAA, HCN, siderophores and availability of phosphorus that may result in better plant (Sunita Mahapatra and Srikanta Das, 2013**,** Sunaina Bisht *et. al.*, 2013, Schroth and Hancock, 1982).

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