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In vitro efficacy of selected potential bioagents against soil borne pathogen *Pythium aphanidermatum*

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Abstract
The Indian Banyan (*Ficus benghalensis*) is perennial plant. Rhizospheric activities of some plants stimulates beneficial microflora which helps in plant growth and disease management. Present investigations were conducted to study *in vitro* antagonistic ability of the microflora isolated from the rhizospheric soil of banyan tree against *Pythium aphanidermatum*. The results revealed that, all the antagonists successfully inhibited growth of test pathogen over untreated control. It was observed that treatment T_3 (*Trichoderma viride*) found most effective showed highest zone of inhibition *i.e.* 69.62% followed by T_2 (*Trichoderma hamatum*) and T_1 (*Trichoderma harzianum*) with percent mycelial inhibition 63.69% and 62.96% respectively. On comparison with bioagents of VNMKV Parbhani isolated strains of *Trichoderma hamatum* and *Trichoderma viride* (63.69% and 69.62%) respectively were better antagonists over available strains of *Trichoderma hamatum* and *Trichoderma viride* (61.47% and 60.47%) respectively. Isolated *Pseudomonas fluorescens* (46.66%) *Pseudomonas striata* (34.07%) *Bacillus subtilis* (38.51%), *Trichoderma koningii* (39.99) are at par to the available strins of *Pseudomonas fluorescens* (47.40%) *Pseudomonas striata* (34.07%) *Bacillus subtilis* (39%), *Trichoderma koningii* (42.21%) respectively.

Keywords: *In vitro*, antagonists, *Pythium aphanidermatum*

Introduction
The Indian Banyan (*Ficus benghalensis*) is perennial plant which belongs to the Kingdom: Plantae Order: Rosales and mulberry family: Moraceae. In Indian tradition *Ficus benghalensis* L. (Banyan tree), *Ficus religiosa* L. (Pipal tree) and *Ficus recemosa* L. (Umbar tree) are worshiped because each and every part these trees is useful in human life. The term rhizosphere is coined by Hiltner in 1904. Hiltner described the rhizosphere as area around a plant root that is inhibited by unique population of microorganisms influenced by the chemicals released from plant roots. It was long known that the rhizosphere activities of some plant stimulates beneficial microorganisms which helps in plant growth and plant disease management. The rhizosphere is the zone where the huge amount microorganism are present which also contains sloughed-off cells and chemicals released by roots provide ready food source for growth. Microbes can make nutrients and minerals in the soil available to plants, produce hormones that spur growth and stimulate plant immune system.
Biological control can achieve the objective of disease suppression through a number of ways such as antibiosis, competition, parasitism, cell wall degradation and induced resistance plant growth promotion and rhizosphere colonization capability. Biocontrol has become an attractive alternative strategy for the management of plant diseases to reduce the excessive use of agrochemicals and its health hazards. Several *Trichoderma* strains applied against a wide spectrum of soil borne pathogens (Philip 2012) [14]. Biological control is less costly and cheaper than any other methods. They give protection to the crop throughout the crop period and does not cause any toxicity to plants and also safer to the environment and to the person who applies them. They can multiply easily in the soil and leave no residual problems. They are very easy to handle and apply to the target (Sharma *et. al.*, 2013) [18].
Now a days the scientists are working in agriculture field are using the soil from the root zone of banyan tree for management of soil borne diseases in fruit crops. Therefore present studies were undertaken to check *In vitro* efficacy of selected potential bioagents against *Pythium aphanidermatum*.

Materials and Methods
Present studies were carried out in the laboratory of Department of Plant Pathology, College of Agriculture, VNMKV, Parbhani. The materials and methods adopted to complete the studies undertaken were described in this chapter under following appropriate heads.

Experimental Materials

The various kinds of materials *viz*., bacteriocides, chemicals, glasswares, culture media and other miscellaneous items required for conducting present studies were obtained from the Department of Plant Pathology, College of Agriculture, VNMKV, Parbhani.

Laboratory facilities

Whole experiment was planned and conducted in the department laboratory, Department of plant pathology, College of Agriculture, Parbhani. *In vitro* studies/experiments were conducted in the laboratory of the Department of Plant Pathology, College of Agriculture, Parbhani.

Methods

Sample Collection

Total 16 soil samples were collected from the rhizosphere of road side banyan tree which were deforested during the widening of road. The soil samples were taken from the depth of 15 cm *i.e*., rhizospheric area of banyan tree. The samples were collected and taken to the laboratory of Department of Plant Pathology, College of Agriculture, Vasantrao Naik Marathwada Agricultural University, Parbhani. For isolation of different micro-organisms and their *in vitro* effects on some soil borne plant pathogens.

Table 1: Details of Location and Month of collection of soil samples for isolation of mycoflora

Sr. No.	District	Tehsil	Location	Number of Samples	Month / Year
1.	Parbhani	Parbhani	Gangakhed Road	1	March 2019
2.	Parbhani	Parbhani	Gangakhed Road	1	March 2019
3.	Parbhani	Gangakhed	Gangakhed Road	1	March 2019
4.	Parbhani	Parbhani	Pathri Road	1	March 2019
5.	Parbhani	Parbhani	Pathri Road	1	March 2019
6.	Parbhani	Pathri	Pathri Road	1	March 2019

Isolation of Micro-organisms

Serial dilution technique was used to isolate micro-organisms from the collected soil samples. These soil samples were air dried in shade and well grind before using for isolation by serial dilutions. Potato Dextrose Agar and Nutrient Agar medium used for isolation and growth. Isolation was carried out under aseptic conditions.

The test tubes then labelled as 10^{-1}, 10^{-2} and 10^{-9}. Each test tube was filled with 9 ml of distilled water. These test tubes were plugged with non absorbent cotton and were sterilized in an autoclave as mentioned earlier. After cooling initial dilution was prepared in test tube labelled as 10^{-1} with the addition of 1 gm soil into the first test tube containing 9 ml of distilled water. This test tube were rolled to and fro between the palms of hands for 5 to 10 minutes for mixing the content uniformally and also to obtain uniform distribution of the soil sample. From the first dilution 1ml of suspension was transferred to the test tube labelled as 10^{-2} containing 9 ml of distilled water with the help of sterilized pipette.

The same procedure was repeated till the original sample was diluted to 10^{-9}. Each time sterilized pipette were used. Then sterilized plates which were heated at 180 °C for 2 hours Hot Air Oven. From these some plates were poured with PDA medium and some with NA medium for isolation of fungus and bacteria respectively. While poring the media was sterilized in Autoclave at 15 lbs and cooled up to 45 °C. Also added antibacterial agents in PDA. The media were poured about 15-20 ml/plate. Then allowed plates to solidify. After that the 1ml suspension from dilution 10^{-3}, 10^{-4} and 10^{-5} for fungus isolation on PDA and from dilution 10^{-7}, 10^{-8}, 10^{-9} for isolation of bacteria NA (while rotating the test tubes in between the palms of hands) were placed on solidified medium and spread it thoroughly on it. Plates were packed with tape and labelled with marker. Plates were incubated in inverted position in BOD incubator at ±28 °C up to 2 days for bacteria and up to 7 days for fungus. The plates were observed every day for growth of micro organisms and bits of their growth from growing colonies were transferred on sterilized PDA slants for fungus and NA slants for bacteria.

Purification of fungal cultures

Isolated cultures of fungi from the rhizosphere sample were transferred on to sterilized PDA media in petri plates under aseptic condition. After the Growth of fungi on plates bits of growing hypha of the fungus were transferred to the PDA slants in the test tube in aseptic condition.

Purification of Bacterial cultures

Isolated cultures of bacteria from rhizosphere sample were transferred on sterilized NA medium on petri plates with the help of inoculating needle having loop at the tip. After the growth of bacterial culture on plates the loop full of suspension from the plates were transferred on NA slants in test tubes.

Maintenance of the cultures

Isolated fungal and bacterial cultures were maintained on sterilized PDA and NA slants respectively in the refrigerator at about 8 to 10 °C. Periodic transfer of the culture were carried out of sterilized PDA and NA slants to keep cultures in active growth.

Culture of the Pathogens

The cultures of the pathogens *viz. Fusarium oxysporum* f. sp. *ciceri, Pythium aphanidermatum, Sclerotium rolfsii* were obtained from the Department of Plant Pathology, VNMKV Parbhani. Which were already identified and tested for pathogenicity.

Mass multiplication of Bio-control agents

Fungal cultures were multiplied on sterilized Potato Dextrose Agar medium in petri plates. For these 20 ml of medium had temperature 45 °C were poured in each 90 mm petri plate. These plates were inoculated with a bit of growth of fungus. The fungal cultures were incubated in an incubator at 28^0± 2 °C temperature for 7 days.

Bacterial cultures were multiplied on sterilized Nutrient Agar Medium in Petri plates for this 20 ml of medium had temperature 45 °C were poured in each petri plate. The plates were inoculated with growing bacterial cultures. These bacterial cultures were incubated in an incubator at 28°± 2 °C temperature for 2 days.

Bio-control agents available at VNMKV, Parbhani

The strains of biocontrol agents available at Department of Plant Pathology, VNMKV Parbhani were also assessed against soil borne pathogens *viz. Fusarium oxysporum* f. sp. *ciceri, Pythium aphanidermatum and Sclerotium rolfsii* by Dual Culture Technique on PDA medium. The isolated biocontrol agents were assessed against soil born pathogen *Pythium aphanidermatum* by Dual Culture Technique on PDA medium as per procedure described by Dennis and Webster (1971). For this procedure 20 ml of PDA media (45^0 C) were poured in each 90 mm diameter Petri plates allowed to solidify. A 5 mm disc (with the help of cork borer) of *Pythium aphanidermatum* as per the need were placed on one end of the medium with the help of sterilized needle. Just opposite to it 5 mm disc of needed biocontrol agent were placed. Control plate also maintained. Then the plates will be incubated at 28±2 °C. On seventh day after incubation, the growth of pathogenic test organisms will be measured and percent growth inhibition was calculated using the formula (Ranjana Chakrabarty *et al*., 2013) [4].

$$\text{Percent Growth inhibition (I)} = \frac{C - T}{C} \times 100$$

Where

I= Percent inhibition over control
C= Radial growth in control
T= Radial growth in treatment

The experimental details

Design: CRD
Replications: 3
Treatments: 9

Results and Discussion

In the present investigations the soil samples for the isolation of microorganisms were collected from the rhizosphere of road sides Banyan trees which were deforested during the widening of road. The soil samples were taken from the location of Gangakhed and Pathri roads of Parbhani District.

Isolation of fungi from rhizosphere of Banyan trees

The fungal species from the rhizospheric soils was isolated by using 10^{-3}, 10^{-4} and 10^{-5} dilutions by serial dilution technique. The results of isolations are presented in Table-2. On the basis of morphology and colony characters, the fungal species were identified. Six samples were collected from the different Banyan trees. The samples were collected from the location of Gangakhed and Pathri road of Parbhani district. The micro-organisms from these samples were isolated on PDA medium by using serial dilution method. These rhizospheric soil samples contained the population of *Trichoderma* spp. and *Aspergillus niger* which were identified on the basis of morphology and colony characters.

The results presented in Table 1. Revealed that, *Trichoderma* spp. were mostly present in collected soil samples. *Trichoderma harzianum* was isolated from the soil sample which was collected from Gangakhed road of Parbhani District. *Trichoderma hamatum* was isolated from another soil sample collected from the location of Gangakhed road from Parbhani Tehsil of Parbhani District. *Trichoderma asperellum* and *Trichoderma koningi* was isolated from the soil sample which was collected from the location of Gangakhed road which comes under Gangakhed Tahsil of Parbhani District. *Trichoderma longibrachiatum* and *Aspergillus niger* was isolated from the soil sample collected from the location of Pathri Road which comes under Parbhani Tehsil of Parbhani District.

Isolation of bacteria from the rhizosphere of Banyan trees

Bacillus and *Pseudomonas* species were isolated from the rhizospheric collected from the rhizosphere of Banyan tree located at Gangakhed and Pathri Road of district Parbhani.

Bacillus subtilis was isolated from the rhizospheric soil samples collected from the location of Gangakhed road which comes under Parbhani Tehsil of Parbhani District. *Pseudomonas fluorescens* was isolated from soil sample collected from the location of Pathri road which comes under Parbhani Tehsil of Parbhani district. *Pseudomonas striata* was isolated from the soil sample collected from the location of Pathri road which comes under Pathri Tehsil of Parbhani District.

The same type of results for isolation of fungal and bacterial spp. from rhizosphere soil were found by workers *viz*. Kannan *et al*., (2009) [9]; Panaiyadian and Chellaia (2011) [12]; Kediugwu *et al*. (2012) [8]; Ahmed *et al.* (2014) [1]; Srinivas *et al.* (2015) [19]; Azizpour and Rouhrazi (2016) [2]; Damam *et al.* (2016) [6] and Rao *et al*. (2016) [15].

Table 2: Collection of soil sample from rhizosphere of Banyan trees

Sr. No.	District	Tehsil	Location	Sample Code
1.	Parbhani	Parbhani	Gangakhed Road	PBN 1
2.	Parbhani	Parbhani	Gangakhed Road	PBN 2
3.	Parbhani	Gangakhed	Gangakhed Road	GGK 3
4.	Parbhani	Parbhani	Pathri Road	PBN 4
5.	Parbhani	Parbhani	Pathri Road	PBN 5
6.	Parbhani	Pathri	Pathri Road	PTR 6

In vitro* evaluation of rhizosphere microflora against *Pythium aphanidermatum

The results obtained on mycelial growth and inhibition of *Pythium aphanidermatum* with isolated bioagents are presented in Table-1 and fig-1 and Plate-I. The results (Table-1 and fig-1) revealed that, all the bioagents exhibited antifungal activity against *Pythium aphanidermatum* and significantly inhibited its growth over untreated control (Plate-I). Among all the bioagents, *Trichoderma asperellum* (T3) found most effective and the test pathogen recorded least linear mycelial growth (27.33 mm) and maximum per cent mycelial inhibition (69.62%) at 7th days after inoculation. The second and third best antagonist found were *Trichoderma hamatum* (T2) and *Trichoderma harzianum* (T1) with mycelial growth 32.66 and 33.33 mm, respectively with per cent mycelial inhibition of 63.69 and 62.96 per cent of the test pathogen, respectively. This was followed by *Pseudomonas fluorescens* (T_7) with colony diameter 54 mm and inhibition 46.66 per cent, *Trichoderma longibrachiatum* (T5) with colony diameter 48.66 mm and inhibition 45.92 per cent, *Aspergillus niger* (T6) with colony diameter 48.66 mm and inhibition 45.92 per cent, *Trichoderma koningi* (T4) with colony diameter 54 mm and inhibition 39.99 per cent, *Bacillus subtilis* (T9) with colony diameter 55.33 mm and inhibition 38.51 per cent. Among all the bioagents treatment, the maximum colony diameter of test pathogen *i.e*. 59.33 mm and least inhibition *i.e*. 34.07 per cent was observed in the

treatment of *Pseudomonas striata* (T8) at 7th days after inoculation. The same results of *in vitro* evaluation of bioagents against *Pythium aphanidermatum* were found by earlier workers. Mishra (2010) [11] screened ten strains of *Trichoderma* against *Pythium aphanidermatum* by dual culture method. The results of the colony interactions clearly demonstrate that *Trichoderma* species exhibited inhibition of the radial growth of *P. aphanidermatum*. The maximum inhibition of *P. aphanidermatum* was by *T. viride*- 14.33 (72.0%), followed by *T. harzianum*- 4572 (69.8%), *T. viride-793* (62.1%), *T. harzianum*- 4532 (60.3%) and *T. virens*- 2194 (59.6%). The least inhibition of *P. aphanidermatum* was recorded by *T. harzianum*- 4 (38.5%) and *T. pseudokoningii*- 2048 (39.3%). Similar reports of *in vitro* evaluation of bioagents against *Pythium aphanidermatum* were reported by workers *viz.* Mishra (2010) [11], Boominathan and Sivakumar (2012) [3], Sheth and Patel (2016) [13] and Chavan *et al.* (2017) [5].

Table 3: *In vitro* evaluation of rhizosphere microflora against *Pythium aphanidermatum*

Tr. No.	Treatments	3rd DAI		5th DAI		7th DAI	
		Col. Dia.* (mm)	% Inhibition*	Col. Dia.* (mm)	% Inhibition*	Col. Dia.* (mm)	% Inhibition*
T1	*Trichoderma harzianum*	16.300	45.663 (42.51)	23.500	60.830 (51.25)	33.333	62.960 (52.51)
T2	*Trichoderma hamatum*	16.600	44.663 (41.93)	21.333	64.443 (53.39)	32.667	63.697 (52.94)
T3	*Trichoderma asperellum*	16.967	43.440 (41.23)	23.000	61.663 (51.74)	27.333	69.623 (56.55)
T4	*Trichoderma koningi*	17.833	49.440 (44.67)	34.000	43.330 (41.16)	54.000	39.997 (39.22)
T5	*Trichoderma longibrachiatum*	15.833	47.220 (43.40)	32.333	46.107 (42.76)	48.667	45.920 (42.65)
T6	*Aspergillus niger*	15.633	47.887 (43.78)	34.000	43.330 (41.16)	48.667	45.920(42.65)
T7	*Pseudomonas fluoresence*	13.000	56.663 (48.82)	37.167	38.050 (38.08)	54.000	46. 660 (43.08)
T8	*Pseudomonas striata*	13.300	55.663 (48.25)	35.000	41.663 (40.20)	59.333	34.070 (35.71)
T9	*Bacillus subtilis*	14.000	53.330 (46.90)	33.167	44.997 (42.12)	55.333	38.517 (38.36)
T10	Control	30.000	--	60.000	--	90	--
S.E. ±		1.455	9.427	1.338	2.304	4.216	4.991
C.D. (P=0.01)		0.490	3.173	0.450	0.776	12.526	14.828

T_1: *Trichoderma harzianum;* T_2: *Trichoerma hamatum;*
T_3: *Trichoderma viride;* T_4: *Trichoderma koningi*;
T_5: *Trichoderma longibactarum;* T_6: *Aspergillus niger*;
T_7: *Pseudomonas fluoresence*; T_8: *Pseudomonas striata*;
T_9: *Bascillus subtilis*; T_{10}: Control

Plate 1: *In vitro* evaluation of rhizosphere microflora against *Pythium aphanidermatum*

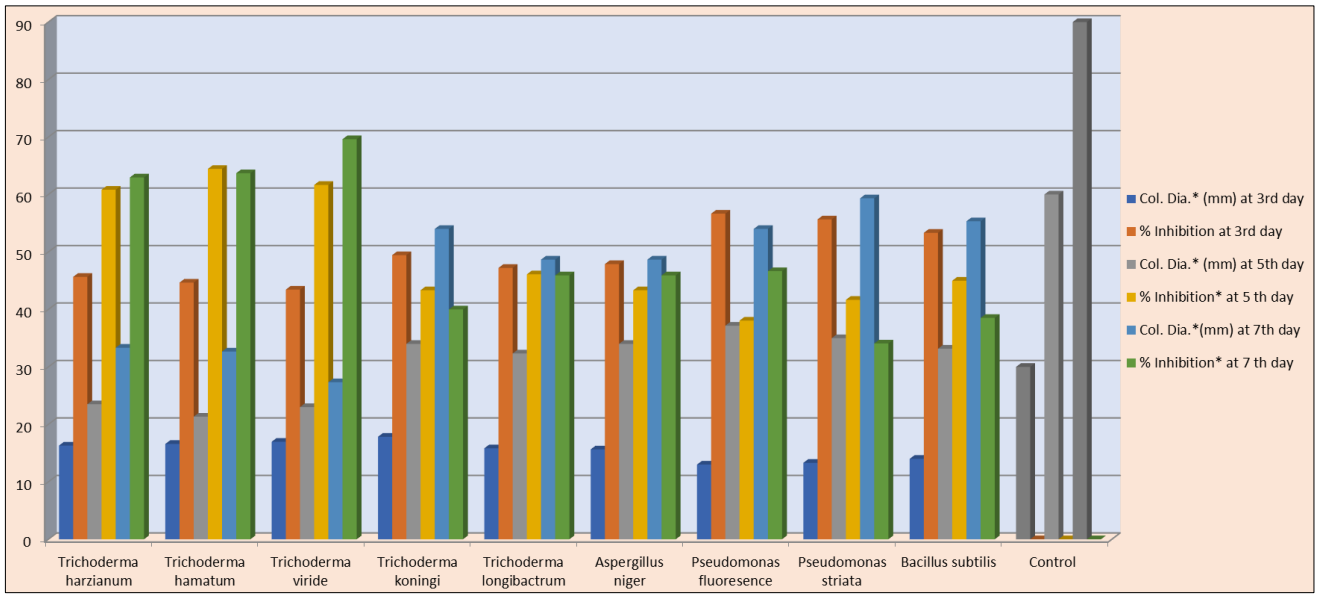

Fig 1: *In vitro* evaluation of rhizosphere microflora against *Pythium aphanidermatum*

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