Biocontrol of *Rhizoctonia* rot of Vanilla (V*anilla planifolia*) using combined inoculation of *Trichoderma* sp. and *Pseudomonas* sp.

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ABSTRACT

Fungal pathogens pose a major problem in causing reduction in yield of vanilla crop to considerable level. *Rhizoctonia solani*, was isolated from naturally infected vanilla plants and an attempt were made to minimize the damage caused by the pathogen using biocontrol agents *Trichoderma harzianum*, and *Pseudomonas fluorescens* isolated from soil. The combined inoculation of *Trichoderma harzianum* with *Pseudomonas fluorescens* treatment showed maximum disease suppression followed by the single inoculation of *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Pseudomonas putida* and *Trichoderma virens* respectively in decreasing order. The results clearly indicated that these biocontrol agents suppressing the disease incidence. Concerning the interaction effect between used antagonistic microorganisms and method of treatments, there was a highly significant effect. These results suggested that using of *Trichoderma harzianum* with *Pseudomonas fluorescens* through soil mixing plus root dipping treatment could be provided not only additional protection against crop loss due to *Rhizoctonia* diseases but also significantly increased vegetative growth of vanilla. The mechanism of biocontrol involved the production of volatile and non volatile organic acids, siderophore, chitinase, peroxidise and salicylic acid. Application of biocontrol agents for crop protection is very significant as it has several advantages such as possibility of multiple pathogen suppression, low cost and promotion of soil fertility.

Keywords: antagonism, bioagents, non volatile organic acids, siderophore, pathogen suppression.

INTRODUCTION

Biocontrol is an effective, ecofriendly and alternative approach for any disease management practice. It has been suggested that microorganisms isolated from the rooted rhizosphere of a specific crop may be better adapted to that crop and may provide better cautious of diseases than organisms originally isolated from other plant species. Such plant associated microorganisms may proved to be better biocontrol agent because they are adapted to rhizosphere effect of particular plant [1]. Vanilla (*Vanilla planifolia* Andrews, Syn. *Vanilla fragrans* saletest Ames) is a herbaceous perennial, climbering orchid (Fa. Orchidaceae). Vanilla is a high value crop [2], which is cultivated for the production of Vanillin (4 - hydroxy3-methoxy benzaldehyde) [3], one of the most valuable flavouring commodities in the food and beverage industry worldwide [4,5]. Vanilla cultivation is severely hampered by the incidence of various diseases. It is susceptible to many fungal diseases such as foot rot and wilting which is caused by *Rhizoctonia oxyspporum* and *Phytophthora* spp., *Sclerotium* rot caused by *Sclerotium rolfsii*, leaf rot, blights and brown spots of anthracnose caused by *Colletotrichum gloeosporioides* [6,7]. Generally application of biocontrol agents simply leads to

inconsistent performance because a single biocontrol agent is not likely to be active against all kinds of soil environments and agricultural ecosystems. Combining different biocontrol agents can contribute to better control of plant pathogens [8]. A positive synergistic interaction between strains of *Trichoderma* sp. bacterial antagonistic such as *Pseudomonas syringae* has been reported for their combined applications in the control of plant pathogens [9]. Hence in recent years, more emphasis is laid on the combined use of biocontrol agents with different mechanisms of disease control, for improved disease control and also to overcome the inconsistent performance of the introduced biocontrol agents [10]. In the present study antagonistic rhizobacteria and *Trichoderma* spp. isolated from the Vanilla rhizosphere were evaluated for their biocontrol potential against *Rhizoctonia* wilt of vanilla.

MATERIALS AND METHODS

Isolation of *Rhizoctonia solani*

Pathogens causing wilt and rot diseases of vanilla namely *Rhizoctonia solani,* was isolated from naturally infected vanilla plants using standard isolation techniques [11].The infected plant parts were collected and brought to the laboratory. The sterilized bits were then placed in sterile Petri dishes containing oatmeal agar medium and incubated at 28 ± 2°C. Mycelial bits were purified by hyphal tip method and transferred to Potato Sucrose Agar (PSA) and Potato Dextrose Agar (PDA) slants and pure cultures of the pathogens were maintained for further studies.

Isolation of *Trichoderma* sp.

The rhizosphere soils of vanilla along with roots were collected from vanilla growing areas in Kerala state and were used for the isolation of *Trichoderma* by serial dilution plate techniques [12], using Martin's rose bengal streptomycin agar medium and malt extract agar medium. For this 10^{-3} and 10^{-4} dilution of soil samples were used. The fungal colonies developed were transferred to PDA medium. Pure culture of fungi were obtained by hyphal tip isolation method and maintained in PDA slants for further studies. *Trichoderma harzianum* obtained from Microbial Culture Collection Centre (MTCC) 801 Chandigarh was used as reference strain.

Isolation of *Pseudomonas* sp.

Pseudomonas sp. was isolated form soil using King's B (KB) agar medium following serial dilution and plating techniques. The plates were incubated at 30°C for 48 hrs. Colonies that came up on KB plates were observed under UV light on a transilluminator. The green fluorescent colonies under UV light were picked up, purified by repeated streaking on the same medium and checked for their fluorescens. *P. fluorescens* obtained from MTCC 1748 Chandigarh was used as reference strain.

Screening of *Trichoderma* sp. and *Pseudomonas* sp against *Rhizoctonia solani*

Antagonistic effect of *Trichoderma* isolates and *Pseudomonas* isolates against *Rhizoctonia solani* were tested by dual culture method outlined by Skidmore, A. M. and Dickson [13]. Three predominant isolates of *Trichoderma* and *Pseudomona* selected from each location were used along with commercial culture of *T. harzianum*. Mycelial discs (6 mm) of pathogen from seven day old culture grown on PDA was inoculated aseptically on one scale of Petri dishes containing PDA and incubated at 28 ± 2°C for 24 hours. After this 6 mm disc of *Trichoderma* isolates were inoculated in the same Petri dishes 3.5 cm away from the pathogen and incubated for 5 days. For *Pseudomonas* it

was inoculated in the same Petri dishes 3.5 cm away from the pathogen and incubated for 5 days. Three replications were maintained for each isolate. Pathogen grown in monoculture served as control. Growth measurements were taken at regular intervals after 24 h of inoculation of antagonistic for four days. Nature of reaction of the antagonist on the pathogen were recorded.

Identification of selected organisms

The pathogen associated with vanilla rot was identified based on the cultural and morphological characters. Cultural characters of the pathogen such as rate of growth, growth pattern etc. in the potato dextrose media were studied. Morphological characters of the pathogen like length of sporangia, L/B ratio, stalk length etc were studied by slide culture technique using lactophenol cotton blue staining. The selected bacterial strains were subjected to cultural, morphological, and biochemical characterization as mentioned in Bergey's Manual of Determinative Bacteriology[14]. 16SrDNA sequencing was done and the sequences were analysed using the gapped BLASTn (www. ncbi.nlm.nig.gov) search algorithm.

Evaluation of biocontrol potential of isolates against fungal pathogens of vanilla under greenhouse condition

A pot culture experiment was conducted to assess biocontrol potential of the isolates *T. harzianum, T. virens, P. fluorescens* and *P. putida* against fungal pathogens of vanilla by dual culture inoculation of the pathogens and biocontrol agents [15]. The trials with vanilla cuttings were carried out in two phases by cross inoculation methods. For seedling inoculation, the aqueous inocula of pathogen and fungal antagonists were prepared by macerating the respective agar cultures in a mixer grinder using distilled water. For bacterial antagonists, the inocula used were the broth cultures. The concentration of the pathogen and antagonists was estimated using dilution plate technique. The experiment was conducted with 27 treatments consisting of 4 isolates, two reference strains, three fungal pathogens and control with only pathogens. The soil was having a pH of 7.2, 0.18%, organic carbon 127 kg/ha available nitrogen, 26 kg/ha of available phosphorous and 346 kg/ha of potassium. The soil had bacterial population of $4.1x10^5$ cfu/g, fungi $3.45x10^3$ cfu/g and actinomycetes $2.54x10^3$ cfu/g. The experiment was conducted with nine treatments with three replications.

RESULTS AND DISCUSSION

The pathogens causing wilt and rot disease of vanilla *Rhizoctonia solani* was isolated from naturally infected vanilla plants using standard isolation technique. The pathogenicity of the organism was proved by following Koch's postulates both under *In vitro* and *In* vivo. A total of 10 *Trichoderma* species were isolated from vanilla rhizosphere by serial dilution plate technique [13], using Martin's rose bengal streptomycin agar medium and malt extract agar medium. Ten rhizobacteria were isolated form vanilla rhizophere soil by serial dilution plate method. Antagonistic effect of *Trichoderma* isolates against *Rhizoctonia solani* were tested by dual culture method outlined by Skidmore and Dickinson (1976) [13].Ten predominant isolates of *Trichoderma* sp. from vanilla growing areas were used. The isolate T5 showed maximum inhibition of 87.76 ± 0.15 followed by the isolate T2 (84.17± 0.38).These two isolates were selected for the further studies (Table 1& Fig.1).

The colony of the isolated pathogenic fungi was cottony pinkish white. The macroconidia are straight to slightly curved, slender, thin walled usually with three or four septa, a foot-shaped basal

cell and a tapered and curved apical cell. The microconidia are ellipsoidal and either have no septum or a single one. The chlamydospores are globose and have thick walls. They are formed from hyphae or alternatively by the modification of hyphal cells.

Table 1. *In vitro* screening of *Trichoderma virens* against *Rhizoctonia solani*.

Biocontrol agent	Percentage of inhibition (after 5days)
T1	44.57 ± 0.29
T2	84.17 ± 0.38
T3	59.78 ± 0.36
T4	65.62 ± 0.34
T5	87.76 ± 0.15
T6	23.36 ± 0.2
T7	68.56 ± 0.28
T8	11.35 ± 0.43
T9	83.00 ± 0.04
T10	13.49 ± 0.17

Note: Values are average of three replicates. Results represented as Mean ± SD

Figure 1. *In vitro* inhibition of *Rhizoctonia solani* by *Trichoderma virens*.

The colonies of the antagonistic fungi were wooly and green. Conidiophores were branched like a pyramidal arrangement. Conidia were unicellular, round or ellipsoidal and were grouped in sticky heads at the tips of the phialides which were the characters of *Trichoderma* spp.The culture T5 showed significant similarity with *Trichoderma virens* based on nucleotide homology and phylogenetic analysis and the culture T2 showed significant similarity with *Trichoderma harzianum.* The sequences were analysed with Basic Local Alignment Search Tool (BLAST) using the program BLASTIN 2.2.24+ NCBI. The sequences generated in this study were deposited in the NCBI gene bank and culture collection centre and got the accession number JN 863298 for the isolate *Trichoderma virens* and JN 000305 for the isolate *Trichoderma harzianum.*

All the 10 isolates of *Pseuodomonas* sp. were tested for their biocontrol potential against the fungal pathogens and the results are presented in table 2. The isolate P7 showed maximum inhibition against *Rhizoctonia solani* (60.24 ± 0.226). The isolate P4 showed 55.41 ± 0.33 inhibition against *Rhizoctonia solani* (Fig.2).

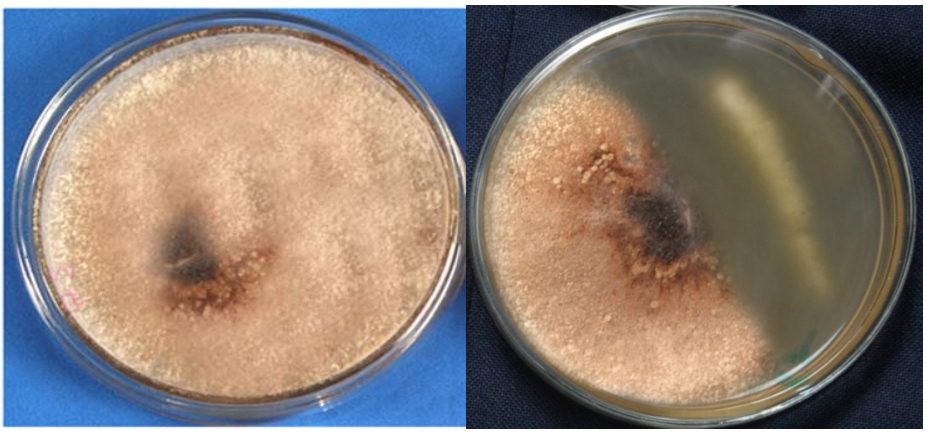

Figure 2. *In vitro* screening of *Pseuodomonas* sp. against *Rhizoctonia solani*.

Table 2. *In vitro* screening of *Pseudomonas* sp against *Rhizoctonia solani*.

Biocontrol agent	Percentage of inhibition (after 5days)
P1	38.58 ± 0.33
P2	46.69 ± 0.14
P3	36.59 ± 0.28
P4	55.41 ± 0.33
P5	43.19 ± 0.35
P6	51.17 ± 0.15
P7	60.24 ± 0.22
P8	48.77 ± 0.36
P9	45.53 ± 0.25
P10	40.21 ± 0.34

Note: Values are average of three replicates. Results represented as Mean ± SD

The rhizobacterial isolates was gram negative motile rods. The organism showed citrate utilization and nitrate reduction. Catalase and oxidase tests were positive. Based on the biochemical reaction the isolate P4 was identified as *Pseudomonas putida* and the isolate P7 was identified as *Pseudomonas fluorescens*. Results of BLAST search of 16S rDNA sequences of the isolate P4 showed close similarity with *Pseudomonas putida* and the isolate P7 showed close similarity with *Pseudomonas fluorescens*. The sequences of the isolated organisms were deposited in the NCBI gene bank and culture collection centre and got the accession number JF701675 for *P. putida* (P4) and JN578642 for the isolate *P. fluorescens* (P7). Based on *in vitro* performance of the *Trichoderma* sp. and *Pseudomonas* sp., four effective antagonistic isolates and two reference strains were screened under pot culture for their biocontrol potential against the fungal pathogen *Rhizoctonia solani* with vanilla as test plant and the results are presented in table 3.

Visible symptoms started to appear from the fifth day after inoculation with *Rhizotonia solani*, in the respective control plants. The symptoms were in the form of leaf yellowing which later turned to leaf rotting. The rotting extended to leaf sheath and rarely to the pseudostem also. Observations were recorded in terms of number of leaves infected and the severity was recorded as the total number of leaves infected in all plants in each treatment. In control plants inoculated with *Rhizotonia solani* alone the infection rate was very high and severity was near 90%. Besides leaf

yellowing and leaf rotting, root rotting followed by wilting and dying of seedlings were also noticed. In all cases, where bioagents were inoculated, disease symptoms were not visible even after 15-20 days after inoculation.

Trichoderma harzianum and *Pseudomonas fluorescens* were proved to be better biocontrol agents (Table 3). *T. virens* and *P. putida* were found to be less effective in controlling fungal disease of vanilla. Single inoculation of *Trichoderma harzianum* were found to be statistically on par with dual applications of *Trichoderma harzianum* and *Pseudomonas fluorescens* where as were statistically superior to the single inoculation treatments with *Pseudomonas fluorescens. Trichoderma harzianum* inoculant was proved to be efficient biocontrol agent than *Pseudomonas fluorescens* in controlling vanilla pathogens (Fig.3).

Table 3. Evaluation of microbial antagonists against *Rhizotonia solani* of vanilla plants.

Treatments	Pre inoculation With bio- control agents	Percentage of leaves infection*
T1	*T.virens*	12.37
T2	*T .harzianum*	8.49
T3	*P.flourescens*	7.18
T4	*P.putida*	20.74
T5	*P.flourescens+T.harzianum*	7.03
T6	*T .harzianum* (std)	8.50
T7	*P. fluorescens* (std)	8.37
T8	*P.flourescens+T.harzianum* (std)	8.42
T9	Control(no biocontrol agent)	90.27
	CD (5%)	1.95

Note: Values are mean of three replicates

The minimum percentage of leaf infection were observed in the combination of *Trichoderma harzianum* with *Pseudomonas fluorescens* treatments. These results support the earlier observations that a combination of biocontrol agents with different mechanisms of disease control will have an additive effect and results in enhanced disease control compared to their individual application. A combination of biocontrol agents is more likely to have a greater variety of traits responsible for suppression of one or more pathogens [16]. Many other studies [17] have reported increased performance in suppression of pathogens or disease by combinations of biocontrol agents. In the same context, Lutz , et al ,[18] reported that the use of bacteria and fungi singly or in combination is a promising approach to improve efficacy of biocontrol treatments. This could be attributed to the involvement of different mechanisms in disease suppression like mycoparasitism, antibiosis or competition for place and nutrients. Our study also support this observation. Evaluation of produced volatile and non volatile compounds showed acceptable performance on inhibiting mycelial growth of pathogens (Table 4).

These results are in confirmation with the reports of several workers who reported the inhibitory effect of volatile compounds produced by *Trichoderma* sp. on several soil borne pathogens [19,20] explained the inhibition of *Rhizoctonia solani* is due to the volatile and non volatile metabolites and cell wall degrading enzymes produced by *Trichoderma* sp. A comparison between the inhibition effects of volatile and non volatile metabolites of *T. harzianum* isolates revealed that the non volatile metabolites seemed to be more effective which is in agreement with the results of the present study.

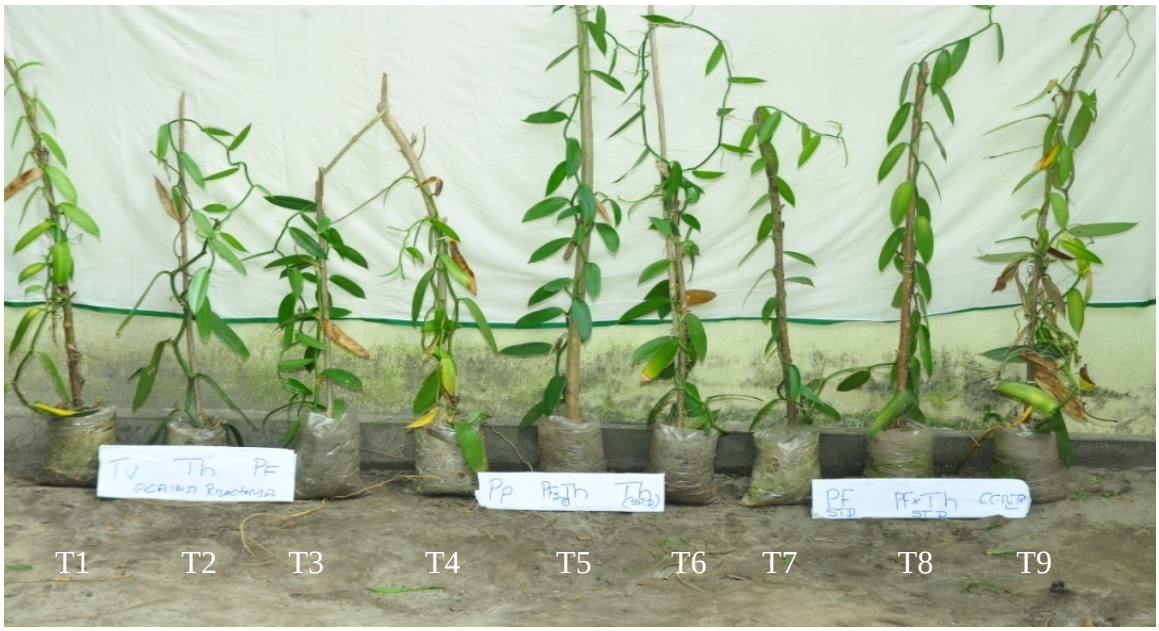

Figure 3. Evaluation of biocontrol potential of isolates against *Rhizoctonia solani* of vanilla plants.

Table 4. Growth reduction of *Rhizoctonia solani* due to Volatile and Non volatile organic compound production by biocontrol agents.

Culture no	% growth reduction due to volatile organic compound production	% o growth reduction due to Non volatile organic compound production
T.virens	49.87	59.12
T.harzianum	78.91	77.77
P.fluorescens	73.21	80.11
P.putida	31.98	31.98
T. harzianum(std)	69.34	69.34
P.fluorescens(std)	68.45	78.45
control	0	0

*Values are average of three replicates.

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