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BIOCONTROL OF FUSARIUM WILT 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. *Fusarium oxysporum*, was isolated from naturally infected vanilla plants and an attempt was 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*, *Trichoderma virens*, respectively in decreasing order. The results clearly indicated that these bio-control agents suppress 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 *Fusarium* 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.

KEY WORDS: Antagonism, Bio-agents, Root rot disease, Siderophore.

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INTRODUCTION

Vanilla (*Vanilla planifolia* Andrews, Syn.*Vanilla fragrans* saletest. Ames) is a herbaceous perennial, climbing orchid (Fa. Orchidaceae). Vanilla is a high value crop (Elizabeth, 2002), which is cultivated for the production of Vanillin (4 - hydroxy3-methoxy benzaldehyde) (Walton *et al .,* 2003), one of the most valuable flavouring commodities in the food and beverage industry worldwide (Besse *et al .,* 2004; Divakaran *et al .,* 2006). 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 *Fusarium oxyspporum* and *Phytophthora* spp.*, Sclerotium* rot caused by *Sclerotium rolfsii*, leaf rot, blights and brown spots of anthracnose caused by *Colletotrichum gloeosporioides* (Thomas *et al .,* 2002; Divakaran *et al .,* 2008).

Biological control of plant pathogens is considered as a potential control strategy in recent years, because chemical control results in accumulation of harmful chemical residues, which may lead to serious ecological problems. Fungi belonging to the genus *Trichoderma* and bacteria such as *Pseudomonas fluorescens ,Bacillus subtilis* are the most promising biocontrol agents against a range of plant pathogens under a variety of environmental conditions (Chet, 1984). *Trichoderma spp*., which is an active myco parasite, has been considered as biocontrol agent of foliar diseases (Elad *et al .,* 1998), and soil borne diseases (Papavizas, 1985). T. harzainum as a bio-control agent through soil mixing plus root dipping treatment could provide not only additional protection against crop loss due to Pythium and Rhizoctonia rot diseases but also has significantly increased vegetative growth, head yield and quality parameters of broccoli plants (Riad *et al .,* 2011). Fusarium wilt is a fungal disease that attacks potato, tomato, Vanilla and pepper. Disease fungi (*Fusarium oxysporum*) enter through the roots and interfere with the water conducting vessels of the plant. As the infection spreads up into the stems and leaves, it restricts water flow, causing the foliage to wilt and turn yellow.

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 (Kamal *et al .,* 2009). A positive synergistic interaction between strains of *Trichoderma* sp. and bacterial antagonistic such as *Pseudomonas syringae* has been reported for their combined applications in the control of plant pathogens (Whipps, 1997). 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 (Latha *et al .,* 2011).In the present study antagonistic rhizobacteria and *Trichoderma* spp. isolated from the Vanilla rhizosphere were evaluated for their biocontrol potential against Fusarium wilt of Vanilla.

MATERIALS AND METHODS

Isolation of Fusarium oxysporum

Pathogens causing wilt and rot diseases of vanilla namely *Fusarium oxysporum,* was isolated from naturally infected vanilla plants using standard isolation techniques (Riker and Riker, 1936). The infected plant parts were collected and brought to the laboratory. The sterilized bits were then placed in sterile petridishes containing oatmeal agar medium and incubated at 28 ± 2^{o}C. Mycelial bits were purified by hyphal tip method and transferred to Potato sucrose Agar (PSA) and Potato dextrose agar (PDA) slands and pure cultures of the pathogens were maintained for further studies.

Isolation of Trichoderma spp.

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 (Johnson and Curl 1972), 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 spp.

Pseudomonas spp. were isolated form soil using King's B (KB) agar medium following serial dilution and plating techniques. The plates were incubated at 30^{o}C for 48 hours. 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 Microbial Culture Collection Centre (MTCC 1748) Chandigarh was used as reference strain.

Screening of Trichoderma spp. against Fusarium oxysporum

Antagonistic effect of Trichoderma isolates against *Fusarium oxysporum* was tested by dual culture method outlined by Skidmore and Dickinson (1976) For this, three predominant isolates of Trichoderma selected form 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 petridishes containing PDA and incubated at 28 ± 2^{o}C for 24 hours. After this 6 mm disc of Trichoderma isolates were inoculated in the same petridishes 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.

Screening of Pseudomonas spp. against Fusarium oxysporum

Antagonistic effect of pseudomonas isolates against *Fusarium oxysporum* was tested by dual culture method outlined by Skidmore and Dickinson (1976) For this, three predominant isolates of pseudomonas selected from each location were used along with commercial culture of pseudomonas. Mycelial discs (6 mm) of pathogen from seven day old culture grown on PDA was inoculated aseptically on one scale of petridishes containing PDA and incubated at 28 ± 2^{o}C for 24 hours. After this *Pseudomonas* was inoculated in the same petridishes 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. 16SrDNA sequencing was done and the sequences were analysed using the gapped BLASTn (www. ncbi.nlm.nig.gov) search algorithm. The sequence was deposited in the NCBI gene bank database.

Cultural and morphological characters of the efficient *Trichoderma* was studied and the characters were compared with description for *Trichoderma*. Cultural characters like growth rate, growth patterns, colour of colony etc. were studied. Morphological characters like mycelium, shape and size of hyphae, conidiophore,

phialede length, nature of phialide were studied by slide culture technique (Rifai, 1969). Molecular identification of *Trichoderma* spp. using Internal transcribed spacer (ITS) region amplification and sequencing. The edited sequences (ITS gene) were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) programme and the sequence was deposited in the NCBI gene bank database.

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 (Ganeshan and Gnana Manickam, 1987). 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 4.1x 10^5 cfu/g, fungi 3.45x 10^3 cfu/g and actinomycetes 2.54x10^3 cfu/g. The experiment was conducted with nine treatments with three replications.

Mechanism of biocontrol activity

A simple plate assay was performed to find out the effect of volatile compounds producedby the isolates. The culture disc(5mm) of *F. oxysporum* was inoculated in the centre of PDA plates. The lid of fungal culture containing plates was removed and the base plate was immediately transferred to the top of the base plate containing the culture of biocontrol agents. It was then sealed tightly with parafilm and incubated for 7-8 days. Control plates with pathogen alone were also maintained. Reduction in hyphal growth of fungal pathogens was calculated.

Hydrogen cyanide production was assessed as per the method Wei et al. (1991). All the antagonistic bacteria were inoculated in King's B medium and siderophore production was checked by $FeCl_3$ test (Neilands 1981). The isolates were grown on succinate medium to test the salicylic acid production. Chitinase enzyme activity was assayed spectrophotometricaly by the method suggested by Boller and Mauch (1988) using colloidal chitin as substrate. The peroxidase activity was estimated using guaiacol as substrate (Hammerschmidt et al. 1982).

RESULTS

Isolation of microbial cultures

The pathogens causing wilt and rot disease of vanilla *Fusarium oxysporum* 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 (Johnson and Curl, 1972) 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 using King's B agar medium

Screening of Trichoderma spp. against Fusarium oxysporum

Antagonistic effect of Trichoderma isolates against *Fusarium oxysporum* were tested by dual culture method outlined by Skidmore and Dickinson (1976). For this, 10 predominant isolates of *Trichoderma* spp from vanilla growing areas were used (Table 1). 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
In vitro screening of Trichoderma virens against Fusarium oxysporum

Sl. No	Biocontrol agent	Percentage of inhibition (after 5days)
1	T1	44.57 ± 0.29
2	T2	84.17 ± 0.38
3	T3	59.78 ± 0.36
4	T4	65.62 ± 0.34
5	T5	87.76 ± 0.15
6	T6	23.36 ± 0.2
7	T7	68.56 ± 0.28
8	T8	11.35 ± 0.43
9	T9	83 ± 0.04
10	T10	13.49 ± 0.17

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

Screening of Pseudomonas spp. against Fusarium oxysporum

All the 10 isolates of *Pseudomonas* spp. 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 *Fusarium oxysporum* (60.24 ± 0.226). The isolate P4 showed 55.41 ± 0.33 inhibition against *Fusarium oxysporum*.

Table 2
In vitro screening of Pseudomonas spp. against Fusarium oxysporum

Serial no	Biocontrol agent	Percentage of inhibition (after 5days)
1	P1	38.58 ± 0.33
2	P2	46.69 ± 0.14
3	P3	36.59 ± 0.28
4	P4	55.41 ± 0.33
5	P5	43.19 ± 0.35
6	P6	51.17 ± 0.15
7	P7	60.24 ± 0.22
8	P8	48.77 ± 0.36
9	P9	45.53 ± 0.25
10	P10	40.21 ± 0.34

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

Identification of selected organisms

The colony of the isolated pathogenic fungi were 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 .

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*

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).

Evaluation of biocontrol potential of isolates against Fusarial wilt of Vanilla under green house condition

Based on *In vitro* performance of the *Trichoderma* spp*.* and *Pseudomonas* spp*.,* four effective antagonistic isolates and two reference strains were screened under pot culture for their biocontrol potential against the fungal pathogen *Fusarium oxysporum* with vanilla as test plant and the results are presented in Table 3.

Table 3
Evaluation of microbial antagonists against Fusarium oxysporum 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 *Values are mean of three replicates

Visible symptoms started to appear from the fifth day after inoculation with *F.oxysporum,* 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 *F.oxysporum*, 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 *Pseudomonas 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* whereas were statistically superior than the single inoculation treatments with *Pseudomonas fluorescens. Trichoderma harzianum* inoculatnt was proved to be an efficient biocontrol agent than *Pseudomonas fluorescens* in controlling vanilla pathogens.

Mechanism of biocontrol activity

The four selected biocontrol agents and the standard reference strain *P. fluorescens* (MTCC 1748) were capable of producing volatile compounds and reduced the growth of the pathogens. The two isolates were found to produce more non volatile organic compounds than the reference strains against *Fusarium oxysporum* (Table 3).

The isolates were found to produce HCN, siderophore and salisylic acid. The isolate P7 showed high siderophore production compared to the isolate P4 and standard culture. In the case of salisylic acid production the isolate P7 showed 0.129 mg of salisylic acid per 50 ml of culture where as the isolate P4 recorded 0.089 mg of salisylic acid per 50 ml of culture.

Chitinase enzyme activity was assayed spectrophotometricaly at 585 nm. The enzyme activity of *Pseudomonas fluorescens* treated plant was 3.5µglcNAc/min/g tissue and control (non treated plant) was 1.5 µg lcNAc/min/g tissue. The peroxidase activity of *Pseudomonas fluorescens* was estimated as 160 units/l and control showed 100 units/l after 45 days.

DISCUSSION

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 (Srivastava *et al .,* 2010). In the present investigation the inhibitory effect of selected antagonistic fungi and bacteria were tested using dual plate culture method (Plate 1,2). Among the fungal antagonists, species of *Trichoderma* are the most potential agents for biocontrol. In this study, ten *Trichoderma* isolates and ten *Pseudomonas* were collected from the Vanilla. *Trichoderma* isolates were identified by their colony and microscopic morphology. The antagonistic activity of *Trichoderma* and pseudomonas were compared by dual culture technique.

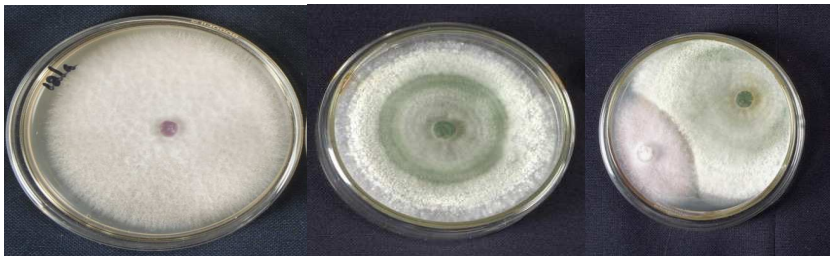

Plate 1
Inhibition of growth of *Fusaruim oxysporum* by T*richoderma harzianum*

Plate 2
Pseudomonas spp against Fusaruim oxysporum

Plate 3
Evaluation of biocontrol potential of isolates against Fusarium oxysporum of vanilla plants

Inducing a plant's own defence mechanism by prior application of a biological agent is an emerging concept and strategy in plant disease management. Disease suppression by biocontrol agents is a result of the sustained manifestation of interactions among the plant, pathogen, biocontrol agent, and environment. Based on the inhibitory efficiency of *In vitro* tested isolates against vanilla pathogens the most effective isolates (*P. fluorescens P. putida ,T. harzianum* and *T. virens*) were selected to be tested under green house. The percentage of reduction of disease incidence are presented in Table 3. Now a days majority of vanilla growers never want to use chemicals for the disease

management even if disease is at its peak because chemical treated beans fetch low price (Suseela Bhai and Dhanesh, 2008).

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 (Duijff *et al .,* 1999). Many other studies (Manjula *et al .,* 2004) have reported increased performance in suppression of pathogens or disease by combinations of biocontrol agents. In the same context, Lutz *et al.,* (2004) 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* spp. on several soil borne pathogens (Kubicek *et al .,* 2001). Rajeswari and Kannabiran (2011) explained the inhibition of *Fusarium oxysporum* is due to the volatile and non volatile metabolites and cell wall degrading enzymes produced by *Trichoderma* spp. A comparison between the inhibiton 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.

Table 4

Growth reduction of Fusarium oxysporum due to the organic acids produced by the isolate

Isolate	% of growth reduction of *Fusarium oxysporum*	
	Volatile organic acid	Non volatile organic acid
T.virens	33.66 ± 0.23	56.23 ± 0.25
T.harzianum	77.88 ± 0.2	81.81 ± 0.58
P.fluorescens	89.03 ± 0.7	80.61 ± 0.55
P.putida	34.10 ± 0.61	34.35 ± 0.38
T. harzianum(std)	39.11 ± 0.46	39.55 ± 0.45
P.fluorescens(std)	58.35 ± 0.55	28.19 ± 0.18
control	4.25 ± 0.44	0

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

P. fluorescence are known to inhibit the growth of plant pathogens by diverse mechanisms such as antibiotic production (Hill *et al .,* 1994), siderphore production (Loper , 1988), HCN release (Voisard et al.,1989) and lytic enzyme release (Fridlender *et al .,* 1993). Hence they have been advocated as ideal biocontrol agents and plant growth promoting rhizobacteria. Induction of resistance by *P. fluorescens* is an additional mechanism by which these bacteria protects several crop plants against pests and diseases (Anand *et al .,* 2010). *P.fluorescens* used in this study were also found to produce volatile and non volatile compounds, siderophore, HCN, salicylic acid,

peroxidase and chitinase enzymes which are attributed to their biocontrol efficiency

CONCLUSION

The present study revealed the antagonistic property of combined inoculation of *Trichoderma* spp. and *Pseudomonas* spp. against Fusarium wilt of Vanilla caused by *Fusarium oxysporum*. The biocontrol agents work by triggering the plant's natural defense system to protect it from more harmful pests and diseases or by competing with pathogens for space and nutrients. These biocontrol agents were also found to produce growth promoting substances, thereby enhancing the growth and bio mass of the Vanilla. In a nutshell the study will be able to endow with us the efficient disease management of Vanilla crop by using biocontrol agents which are very cost effective and eco-friendly in the present contest of sustainable agriculture.

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