INTRODUCTION

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. At present, effective management of plant diseases & microbial contamination in several agricultural commodities is generally achieved by the use of synthetic pesticides. However, the incessant & indiscriminate application of these chemical fungicides has caused health hazards in animals & humans due to residual toxicity. In recent years, large number of synthetic fungicides have been banned in the western world because of their undesirable attributes such as high & acute toxicity. Many pathogenic microorganisms have developed resistance against chemical fungicides. This seriously hinders the management of diseases of crops & agricultural plants. Considering the deleterious effects of synthetic fungicides on life supporting systems, there is an urgent need for alternative agents for the management of pathogenic microorganisms.

Although the value of eco-friendly pest management in sustainable agriculture has been well recognized, only very little is being adapted at field level. This eco-friendly pest management gives greater emphasis for the usage of biological control. Bio-control methods are successful in non-chemical and eco-friendly approach in the sustainable agricultural production. Fungi belonging to the genus *Trichoderma* and bacteria such as *Pseudomonas, Bacillus subtilis* are the most promising bio control agent against a range of plant pathogens under a variety of environmental conditions (Chen, et al, 1995). Among the microbial bio-control agents *Pseudomonas fluorescens* and *Trichoderma harzianum* are used for the management of most of the disease management of Crops.

Trichoderma spp. are a group of antagonistic cellulolytic fungi, capable of controlling a number of diseases of plants. They are considered as biocontrol agent of foliar diseases and soil borne diseases (Papavizas, 1985). Many species of *Trichoderma* namely *Trichoderma harzianum* and *Trichoderma viride, Trichoderma virens* etc, isolates from rhizosphere were found to have good antagonistic potential against many soil born fungi, such as *Fusarium oxysporum, Sclerotium rolfsii,*

Rizoctonia solani. As an antagonist, *Trichoderma* spp. produce antibiotics and antifungal toxic metabolites viz Trichodermin, viridin, etc and also inhibit pathogens by secreting enzymes like glucanase, cellulase, chitinase, protease etc, which disintegrate the cell wall of pathogen and act as a competetor for mineral nutrients.

Pseudomonas spp. are also used as a potential bio-control agent which are major group of bacteria found surviving in the rhizosphere of most of the field crops . They are also known as plant growth promoting rhizobacteria [PGPR], as they promote plant growth by secreting auxins, giberllins, cytokines. etc. *Pseudomonas fluorescens, Pseudomonas putida*, *Pseudomonas cepacia* are the important bio-control agents and they produce antibiotics like Pyrol, Nitrin, Oomycin-A etc and Hormones like Indol acetic acid, Giberlic acid, and Siderphores produced by these bacteria inhibit the growth of pathogen.

Vanilla [*Vanilla planifolia*] belongs to the family ***Orchidaceae*** ,advanced group of Monocotyledons. The family is one of the largest flowering plants with, about 700 genera and 2000 species. Vanilla has a real economic value in the food and related industries, owing its unique flavour and pleasant aroma, other counter parts being well known for its most attractive flavours. The substance chiefly responsible for the fragrant, flavour, and pleasant aroma of the Vanilla beans is **Vanillin.** Vanilla essence is largely used in the preparation of ice creams, Chocolates, bakery products, puddings, pharmaceuticals, liquors, and perfumes. In India the production of Vanilla was estimated to be 10 tones during 2000- 2005, and a recent survey puts it at 1600 hectors with 60 tones. A number of pathogenic fungi and bacteria cause diseases in Vanilla and some of which result in to the total death of the plant. The pathogen affect almost all the plant parts like root, stem, leaves, and beans. Species of *Fusarium, Sclerotium, Rizoctonia, Phytophthora and Colletotricum* are the commonly occurring pathogens, which cause series damages, (Joseph Thomas et.al, 2005).

Generally application of bio-control agents simply leads to inconsistent performance because a single bio-control agent is not likely to be active against all

kinds of soil environments and agricultural eco-systems. In such conditions the use of combination of strains which have different modes of action will be more useful to improve suppression. A combination of *Pseudomonas fluorescens and Trichoderma harzianum* were having more effect on the control of disease of vanilla

In the light of the above literature, the present study was undertaken with the following objectives

1. Isolation, screening and identification of effective bio-control agents such as *Pseudomonas fluorescens and Trichoderma harzianum* against major plant pathogens of vanilla(*Vanilla planifolia*)

2. To study the mechanisms of the bio control activity and the production of plant growth promoting substances by these isolates

3. To study the effect of the isolated bio control agents on the growth and nutrient uptake of vanilla plants

4. Pot culture experiment to check the efficacy of the bio control agents against vanilla pathogens

5. Colonization study using green fluorescent protein (GFP) as a marker.

MATERIALS AND METHODS

Isolation of Pathogens and biocontrol agents

The major phytopathogens of vanilla namely *Fusarium oxysporum, Rizoctonia* solani, *and Sclerotium rolfsii* were isolated from naturally infected plants using standard isolation techniques and pure cultures of the pathogens were maintained for further studies. The pathogenocity of the organisms were proved by Koch's postulates

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* species and *Psuodomonas fluorescens.*

Screening of microbial antagonists against phytopathogens

Antagonistic effect of *Trichoderma* isolates and the rhizobacteria *Pseudomonas* spp. against *Fusarium oxysporum Rizoctonia solani* and *Sclerotium rolfsii* were tested by dual culture method outlined by Skidmore and Dickinson (1976) .Percent inhibition of mycelial growth of the pathogen was calculated using the formula suggested by Vincent and Budge (1990).

Identification of Pathogens

The pathogens were identified based on the cultural and morphological characters..Morphological characters of the pathogen like length of sporangia, L/B ratio, stalk length etc were studied by slide culture technique. The identification of Fungi was confirmed by amplification of ITS region and sequencing.

Identification of *Trichoderma* and *Pseudomonas* sp.

Species of *Trichoderma* and *Pseudomonas* sp. selected from screening test were identified based on cultural and morphological characters and Identification of Fungi was confirmed by amplification of ITS region and sequencing. The *Pseudomonas* sp. were identified molecularly by 16S rDNA sequencing.

Compatibility Studies

Compatibility studies were carried out to find out whether the selected antagonists were compatible with the commonly used chemicals recommended against vanilla pathogens. Compatibility of *Trichoderma* isolates with antagonistic *Pseudomonas* sp. were also tested.

Screening of bio control agents for Growth enhancement of vanilla

A pot culture experiment was carried out in green house to check the efficacy of the biocontrol agents *Trichoderma virens,Trichoderma harzianum, Psuodomonas fluorescens, Pseudomonas putida, Trichoderma harzianum* (Standard), and *Psuodomonas fluorescens*(Standard), for Growth enhancement of vanilla crop.Biometric observations such as Length of vine, Number of leaves, Fresh weight

of shoots, Fresh weight of roots, Dry weight of shoot, Dry weight of roots and Nutrients uptake were recorded.

In vivo experiment to check the biocontrol efficacy of the antagonists against phyto pathogens

For testing the biocontrol activity of these antagonists, experiments were carried out in vanilla cuttings under green house conditions. 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 using a mixer grinder with distilled water. For bacterial antagonists, broth cultures were used . The concentration of the pathogen and antagonists were estimated using dilution plate technique.

In phase 1, aqueous inocula of the bio agents were first drenched at the base of the cuttings followed by cross inoculation with the pathogens, *Fusarium .oxysporum, Rizoctonia solani*, and *Sclerotium rolfsii* in the respective treatments. In phase 2, aqueous suspension of the pathogen was first drenched at the base of the cuttings and then it was followed by cross inoculation with the antagonists after seven days.

Mechanisms of Biocontrol

Mechanisms of biocontrol such as Volatile organic compound production, Non Volatile organic compound production, HCN production, Siderophore production, Salicylic acid production, Chitinase enzyme production and Assay of Peroxidase (PO) were studied using standard protocol . Colonization study was carried out by using green fluorescent protein (GFP) as a marker

RESULTS AND DISCUSSION

Isolation of Pathogens and bio control agents

The pathogen causing rot and wilt diseases of vanilla, *Fusarium oxysporum Rizoctonia solani*, and *Sclerotium rolfsii* were isolated . The pathogenocity of the organism associated with various diseases of vanilla was proved by Koch's postulates

Screening of microbial antagonists against phytopathogens of vanilla

Twenty nine isolates of *Trichoderma* and twenty *Pseudomonas* sp. *were isolated* .Antagonistic effect of *Trichoderma* isolates and *Pseudomonas* sp. against *Fusarium oxysporum, Rizoctonia solani* and *Sclerotium rolfsii* were tested by dual culture method and percentage of inhibition was calculated . The isolates Tv5 showed (77.77% inhibition against *Fusarium oxysporum* ,87.88% aginst *Rizoctonia solani* and 80.67% against *Sclerotium rolfsii* ,Th2 showed 83.88% inhibition against *Fusarium oxysporum* ,89.34% aginst *Rizoctonia solani* and 85.87% against *Sclerotium rolfsii*)and Pf7 showed 60.23% inhibition against *Fusarium oxysporum* , 58.67% aginst *Rizoctonia solani* and 54.19% against *Sclerotium rolfsii* Theses isolates were selected for further studies .

Identification of Pathogens

The pathogens were identified based on the cultural and morphological characters as *Fusarium oxysporum, Rizoctonia solani* and *Sclerotium rolfsii*

Identification of Trichoderma and antagonistic Rhizobacteria

Species of *Trichoderma* and *Pseudomonas* sp. selected from screening test Tv5,Th2 and Pf7 were identified as *Trichoderma virens, ,Trichoderma harzianum,* (JN000305) , *Pseudomonas flour*scens (JN578642) ,respectively by amplification of ITS region and 16sr DNA sequencing

Compatibility Studies

Compatibility studies showed that the selected antagonists were not compatible with the commonly used chemicals. *Trichoderma* isolates were Compatible with *Pseudomonas* sp. and hence can be used combinely

Screening of biocontrol agents for Growth enhancement in vanilla

The maximum length of vine was observed in the combination of *Trichoderma harzianum and Pseudomonas fluorescens* and followed by *Trichoderma harzianum*, and *Pseudomonas fluorescens* individually. But *Trichoderma virens* showed a less effects in the length of vine of vanilla after 165 days . The maximum number of leaves were observed in the treatment were the combination of *Trichoderma harzianum* and *Pseudomonas fluorescens* used and then *Pseudomonas fluorescens, Trichoderma harzianum, Pseudomonas putida, Trichoderma virens* respectively in decreasing order. Control with no biocontrol agents showed minimum number of leaves of vanilla .

The maximum fresh weight of shoots and roots were observed in the dual inoculation of *Trichoderma harzianum* and *Pseudomonas fluoerscens* and then *Pseudomonas fluorescens* followed by *Trichoderma harzianum, Pseudomonas putida. Trichoderma virens* in decreasing order of fresh and dry weight of both shoots and roots after 165 days of inoculation. Nutrients uptake of N.P. and K in plant samples were analysed after five planting. The maximum uptake of all elements were observed in the treatment were the combination of *Trichoderma harzianum* and *Pseudomonas fluorescens* used and then *Pseudomonas fluorescens ,Trichoderma harzianum, Pseudomonas putida ,Trichoderma virens* respectively in decreasing order . Control with no biocontrol agents showed minimum uptake of NPK elements

***In vivo* experiment to check the bio control efficacy of the antagonists against phyto pathogens**

Visible symptoms started to appear from the fifth day after inoculation with *Fusarium oxysporum ,Rizoctonia solani* and *Sclerotium rolfsii*. 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 pseudo stem also. Observations were recorded in terms of the 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 *Fusarium oxysporum, Rizoctonia solani* and *Sclerotium rolfsii* 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 first(in first phase experiment), and later cross inoculated withpathogens,the disease symptoms were not visible even after 15 to 20 days. In treatments where the pathogen was inoculated first(in second phase experiment) and later cross inoculated with bio control gents, disease symptoms were observed and in some cases the seedling collapsed.

The minimum percentage of leaf infection were observed in(in both phase of experiment) the dual inoculation of *Trichoderma harzianum* with *Pseudomonas fluorescens* . *Pseudomonas fluorescens* showed less infection rate over *Trichoderma harzianum* against *Sclerotium rolfsii* in phase two. *Trichoderma harzianum* showed less infection rate over *Pseudomonas fluorscens* against *Rizoctonia solani* in both phases . *Trichoderma harzianum* showed less infection rate over *Pseudomonas fluorescens* against Sclerotium rolfsii in phase one but in phase two *Pseudomonas fluorescens* showed less infection rate over *Trichoderma harzianum* against *Sclerotium rolfsii.Trichoderma virens* showed less infection rate over *Pseudomonas putida* against all phytopathogens in both phases . Control with no biocontrol agents showed maximum percentage of leaf infection of vanilla in both phases.

Mechanisms of Biocontrol

Both Volatile and Non Volatile organic compound production by *Trichoderma virens, Trichoderma harzianum* and *Pseudomonas fluorescens* inhibited the growth of all phytopathogens .HCN production was studied in *Pseudomonas fluorescens* . *Pseudomonas fluorescens* produced Siderophore Salicylic acid and peroxidase enzyme. *Trichoderma virens and Trichoderma harzianum* produced the Chitinase enzyme

Colonization study using green fluorescent protein (GFP) as a marker

The antagonistic endophyte *Pseudomonas fluorscens* was successfully transformed with pGFPuv. The transformed colonies in the LB-Ampicillin plate exhibited green fluorescence when viewed in U.V. light.

Green fluorescent endophytic bacteria were observed within the plant tissue when cross sections of the petiole of treated vanilla were viewed under the Confocal laser scanning microscope. The bacteria were found to be localized within the intercellular spaces of the tissue.

Discussion

The present study revealed the antagonistic property of *Trichoderma sp.*and *P. fluorescens* against *Fusarium oxysporum,, Rhizoctonia solani* and *Sclerotium rolfsii*. The biocontrol agents work in by triggering the plant's natural defence system to protect it from more harmful pests and diseases or by competing with pathogens for space and nutrients. Because these bacteria and fungi are not toxic to other organisms, they pose a low risk to the environment.

Conclusion

The above proposed study will provide us in depth knowledge on the influence of the Bio-control agents like *Pseudomonas fluorescens* and *Trichoderma harzianum* on the control of the various diseases of Vanilla crop biologically. The beneficial side of the studies will be a great boon to the vanilla growers of Kerala state. These Bio-control agents were also found to produce growth promoting substances, thereby enhancing the growth and bio mass of the Vanilla. In short the study will able to provide us the efficient disease management of Vanilla crop by using Bio-control agents which are very cost effective and eco-friendly in the present contest of sustainable agriculture.