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Combined Inoculation of *Pseudomonas fluorescens* and *Trichoderma harzianum* for Enhancing Plant Growth of Vanilla (*Vanilla planifolia*)

A.R. Sandheep, A.K. Asok and M.S. Jisha
School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills P.O. Kottayam, Kerala, 686560, India

Abstract: This study was conducted to evaluate the plant growth promoting efficiency of combined inoculation of rhizobacteria on Vanilla plants. Based on the *in vitro* performance of indigenous *Trichoderma* spp. and *Pseudomonas* spp., four effective antagonists were selected and screened under greenhouse experiment for their growth enhancement potential. The maximum percentage of growth enhancement were observed in the combination of *Trichoderma harzianum* with *Pseudomonas fluorescens* treatment followed by *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Pseudomonas putida* and *Trichoderma virens*, respectively in decreasing order. Combined inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* registered the maximum length of vine (82.88 cm), highest number of leaves (26.67/plant), recorded the highest fresh weight of shoots (61.54 g plant^{-1}), fresh weight of roots (4.46 g plant^{-1}) and dry weight of shoot (4.56 g plant^{-1}) where as the highest dry weight of roots (2.0806 g plant^{-1}) were achieved with treatments of *Pseudomonas fluorescens*. Among the inoculated strains, combined inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* recorded the maximum nitrogen uptake (61.28 mg plant^{-1}) followed by the combined inoculation of *Trichoderma harzianum* (std) and *Pseudomonas fluorescens* (std) (55.03 mg plant^{-1}) and the highest phosphorus uptake (38.80 mg plant^{-1}) was recorded in dual inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens*.

Key words: Dual inoculation, greenhouse experiment, nitrogen uptake, phosphorus uptake, rhizobacteria

INTRODUCTION

Vanilla (*Vanilla planifolia* Andrews, Syn. *Vanilla fragrans* saletest. Ames) is a herbaceous perennial, climbing orchid (Family Orchidaceae). Vanilla is the second most expensive spice after Saffron and is still big money spinners. It is often referred to as green gold and princess of spices. In India it is grown in an area of 2545 hectares covering Karnataka, Kerala and Tamilnadu with production of about 100 metric tons (Kuruvilla *et al*., 2004).

The recognition of Plant Growth-promoting Rhizobacteria (PGPR), a group of beneficial plant bacteria, as potentially useful for stimulating plant growth and increasing crop yields has evolved over the past several years. The *Pseudomonas fluorescens* have emerged as the largest and potentially most promising group of PGPR through several mechanisms viz., antibiotics production, siderophore production, HCN release (Yu *et al*., 2011), induction of systemic resistance and competitive colonization of plant roots (Viswanathan and Samiyappan, 2002). Hence, they have been advocated as ideal biocontrol agents and plant growth promoting rhizobacteria. In addition to the ability of *Trichoderma* spp. to attack or inhibit the growth of plant pathogens directly, recent discoveries indicate that they can also induce systemic and localized resistance to plant pathogens (Harman, 2000). Moreover, certain strains also have substantial influence on plant growth and development. Their enhancement of plant growth has been known for many years and can occur in both auxenic systems (Yedidia *et al*., 2001) and natural field soils (Rojo *et al*., 2007).

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. A positive synergistic interaction between strains of bio inoculants may have additive effects on their plant growth stimulating activity. Hence in this study the dual inoculation of *Pseudomonas fluorescens* and *Trichoderma harzianum* was checked for their additive plant growth enhancement of Vanilla (*Vanilla planifolia*).

Corresponding Author: M.S. Jisha, School of Biosciences, Mahatma Gandhi University, Priyadarshini Hills P.O. Kottayam, Kerala, 686560, India Tel: +919497664697 Fax: 91-481-2731002

MATERIALS AND METHODS

Cultures: Two bacterial isolates identified as *Pseudomonas fluorescens, Pseudomonas putida* and two fungal cultures identified as *Trichoderma virens, Trichoderma harzianum* isolated from rhizosphere soil of Vanilla plants were used in this investigation. Two reference strains *Trichoderma harzianum* (MTCC 801) and *Pseudomonas fluorescens* (MTCC 1748) were also used.

Preparation of microbial cultures: For inocula preparation, the antagonistic bacterial isolates were grown separately in Tryptic Soy Broth (TSB) and incubated at 28°C for three days. Bacterial cells were harvested by centrifugation (7,000 rpm for 20 min). After removal of the culture medium, the bacterial pellet was washed in sterile water and centrifuged again (7,000 rpm for 20 min). Bacterial cells were then resuspended in sterile saline solution and cell density was adjusted to get approximately 8×10^9 CFU mL^{-1}.

Sporulated pure fungal cultures prepared on PDA medium were selected for the preparation of spore suspensions from each fungal isolate. A total volume of 20 mL sterile water was spread in aliquot on a culture plate and the fungal colony surface was lightly scraped using a sterile spreader. The cultures were filtered through Whatman No. 42 filter paper in to a sterile glass bottle. Spore counts were taken using a haemocytometer and the suspension was adjusted to have approximately 1×10^7 spores per 1 mL.

Green house evaluation: A pot culture experiment was conducted to the plant growth promotional activity of the isolated antagonists *Trichoderma virens, Trichoderma harzianum*, *Pseudomonas fluorescens*, *Pseudomonas putida* and the reference strain *Trichoderma harzianum* (MTCC 801) and *Pseudomonas fluorescens* (MTCC 1748). Three nodded vanilla cuttings were planted in sterilized soil, sand, cow dung mixture in 1:1:1 ratio. The sterilized potting mixture were filled in 6×8 inch polybags @ 1.5 kg/bag. The potting mixture without inoculation served as control. The soil was having a pH of 7.6, 0.18% organic carbon, 134 kg ha^{-1} of available nitrogen, 15 kg ha^{-1} of available phosphorous and 267 kg ha^{-1} of potassium. The soil had bacterial population of 3.7×10^5 CFU g^{-1}, fungi 3.98×103 CFU g^{-1} and actinomycetes 1.67×10^3 CFU g^{-1}. The experiment was conducted with nine treatments and three replication.

Plant growth parameters such as length of vine, number of leaves, fresh weight of shoots, fresh weight of roots, dry weight of shoot and dry weight of roots were recorded 45 days after planting and later at fortnightly intervals up to five and half months.

Nutrient analysis: Nutrients like N and P in plant samples were analyzed after five and half months of planting. For this, the whole plant was uprooted, washed off soil particles and dried in an oven at 60°C. After drying, samples were powdered and the fine powder was used for estimation of nutrient elements. N and P were analyzed according to the procedure given by Jackson (1973).

RESULTS

Based on the *in vitro* performance of *Trichoderma* spp. and *Pseudomonas* spp. four effective antagonists were selected and screened under greenhouse experiment for their growth enhancement potential (Fig. 1).

Length of vine (cm) of vanilla at 165 days after planting (DAP) as influenced by different treatments is listed in Table 1. The maximum length of vine (82.88 cm) was observed in the combination of *Trichoderma harzianum* and *Pseudomonas fluorescens* treatment and was significantly higher over the rest of the treatments but was on par with the combined inoculation treatment of reference strains (81.20). The uninoculated control treatment recorded the least vine length of 51.87 cm.

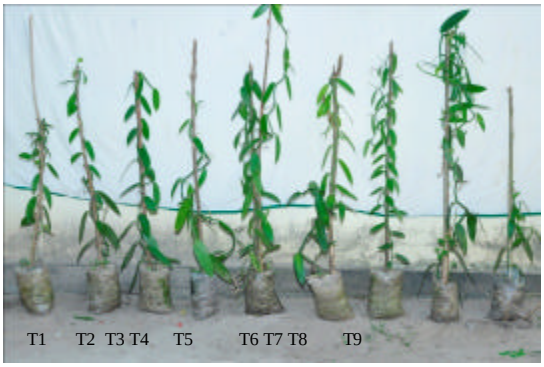

Fig. 1: Plant growth promoting activity of *Trichoderma* spp. and *Pseudomonas* spp. inoculated to vanilla crop, T1: *T. virens*, T2: *T. harzianum*, T3: *P. fluorescens*, T4: *P. putida*, T5: *T. harzianum*+*P. fluorescens*, T6: *T. harzianum* (std), T7: *P. fluorescens* (std), T8: *T. harzianum* (std)+*P. fluorescens* (std), T9: Control

Table 1: Influence of antagonistic *Trichoderma* and *Pseudomonas* species on plant growth parameters of vanilla (After 165 days)

Treatments	Length of vine (cm)	No. of leaves
T. virens	55.92	20.33
T. harzianum	77.47	22.67
P. fluorescens	73.02	24.67
P. putida	63.03	22.33
T. harzianum+ *P. fluorescens*	82.88	26.67
T. harzianum (std)	73.17	24.67
P. fluorescens(std)	72.68	24.00
T. harzianum(std)+ *P. fluorescens* (std)	81.20	25.67
Control	51.87	21.67
CD(5%)	2.11	N.S
VR	**234.6081	**1.897727

*Values are mean of three replicates

Table 2: Effect of applications of *Trichoderma* and *Psudomonas* isolates on the biomass of vanilla (After 165 days)

Treatments	Fresh weight of shoot (g plant^{-1})	Fresh weight of root (g plant^{-1})	Dry weight of shoot (g plant^{-1})	Dry weight of root (g plant^{-1})
T. virens	49.27	4.41	3.33	1.01
T. harzianum	54.40	4.19	4.24	1.13
P. fluorescens	58.54	4.19	4.42	2.08
P. putida	51.69	4.09	4.15	1.25
T. harzianum+ *P. fluorescens*	59.11	4.46	4.56	1.88
T. harzianum (std)	55.91	4.03	4.06	1.13
P. fluorescens (std)	56.59	4.29	4.37	1.44
T. harzianum (std)+ *P. fluorescens* (std)	61.54	4.21	4.43	1.68
Control	42.97	3.67	3.21	1.01
CD(5%)	10.5	ns	ns	ns
VR	**2.619628	**0.097505	**0.787807	**1.626945

*Values are mean of three replicates. NS: Not significant

Number of leaves per plant of vanilla was counted after 165 days of planting (Table 1). highest number of leaves (26.67 leaves/plant) and was superior over the rest of the treatments. The next effective treatment in number of leaves per plant was dual inoculated *Pseudomonas fluorescens* and Trichoderma *harzianum* (std) with 25.67 leaves/plant. Plants with uninoculated control treatments recorded 21.67 leaves/plant.

Among the bio-inoculants tested combined inoculation of *Trichoderma harzianum* (std) and *Pseudomonas fluorescens* (std) recorded the highest fresh weight of shoots (61.54 g plant^{-1}) which was statistically on par with the treatment of isolated *Trichoderma harzianum* and *Pseudomonas fluorescens* (59.11 g plant^{-1}) (Table 2). The combined inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* showed the highest fresh weight of roots (4.46 g plant^{-1}) which was statistically on par with the treatments *Pseudomonas fluorescens* (std) (4.29 g plant^{-1}) and combined inoculation of *Trichoderma harzianum* (std) and *Pseudomonas fluorescens* (std) (4.21 g plant^{-1}) and was superior over all other treatments (Table 2).

Table 3: Nutrient up take (mg plant^{-1}) by vanilla crop as influenced by biocontrol agents

Treatments	Nitrogen uptake (mg plant^{-1})	P uptake (mg plant^{-1})
T. virens	28.38	14.56
T. harzianum	40.99	19.28
P. fluorescens	45.13	28.42
P. putida	29.84	15.85
T. harzianum+*P. fluorescens*	61.28	38.80
T. harzianum (std)	43.22	14.19
P. fluorescens (std)	33.28	22.81
T. harzianum (std) +*P. fluorescens* (std)	55.03	34.51
Control	18.01	15.80
CD (5%)	7.48	3.94
VR	**29.20747	**48.12689

*Values are mean of three replicates

The dry weight of shoot in vanilla plants was highest (4.56 g plant^{-1}) in plant receiving the combined inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* (Table 2). Among the isolates *Pseudomonas fluorescens* exhibited highest dry matter content (4.42 g plant^{-1}) which was equal to the treatment receiving the dual inoculation of reference strains (4.43 g plant^{-1}). The highest dry weight of roots was recorded in vanilla plants inoculated with *Pseudomonas fluorescens* (2.080 g plant^{-1}) closely followed by the combined inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* (1.889 g plant^{-1}) (Table 2).

Data presented in Table 3 indicated that there were significant differences in the nitrogen and phosphorus uptake by vanilla. Among the inoculated strains, combined inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* recorded the maximum nitrogen uptake (61.28 mg plant^{-1}) followed by the dual inoculation of *Trichoderma harzianum* (std) and *Pseudomonas fluorescens*(std) (55.03 mg plant^{-1}) which were on par with each other but both were significantly superior over rest of the isolates including the reference strains. The highest phosphorus uptake (38.80 mg plant^{-1}) was also recorded in dual inoculation of *Trichoderma harzianum* and *Pseudomonas fluorescens* followed by the combined inoculation of *Trichoderma harzianum* (std) and *Pseudomonas fluorescens* (std) (34.51 mg plant^{-1}) both of which were significantly superior over all other strains and they also differed significantly among themselves (14.56 g plant^{-1}) which was statically on par with the uninoculated control (15.80 g plant^{-1}).

DISCUSSION

Vegetative growth parameters of vanilla expressed as length of vine, number of leaves per plant and biomass as affected by different bio-inoculants were evaluated under

green house conditions in a pot culture experiment (Table 1, 2). Data presented clearly demonstrated that there were significant differences in the vegetative growth parameters due to the inoculation of isolated bio-inoculants. The obtained results are in harmony with that of Srivastava *et al.* (2010) on tomato and Esitken *et al.* (2010) on strawberry. All these workers reported that using beneficial microorganisms as biocontrol agents led to enhancement of plant growth parameters. Such enhancement may be due to induce plant resistance (De Meyer *et al*., 1998), production of extracellular enzymes and antifungal or antibiotics, which reduce the negative effect of biotic stress on plant and produce growth promoting substances (Szczech and Shoda, 2004).

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield. The increased growth and biomass of crop plant as a result of inoculation with PGPR strains has been previously reported (Esitken *et al*., 2010). From the results it might be suggested that antagonistic isolates played an important role in stimulating root growth there by enhanced the nutrient uptake which in turn increased the plant growth (El-Mohamedy *et al*., 2011). The antagonistic *Pseudomonas fluorescens* are also known to produce plant growth promoting substances such as indole acetic acid and gibberllic acid solubilises insoluble phosphate (Suneesh, 2004; Megha, 2006) which might have resulted in increased plant growth.

Trichoderma harzianum and *Trichoderma viride* are active rhizosphere colonizers and the fungi produce antibiotics such as gliotoxin, viridin, cell wall degrading enzymes and biologically active heat stable metabolites such as ethyl acetate (Khan *et al*., 2004). These substances are involved in disease suppression and plant growth promotion. *Trichoderma* spp. is reported to give systemic protection against many pathogens (Hanson, 2000) and is also known to provide plants with useful molecules such as glucose oxidase and growth stimulating compounds that can enhance the plant growth (Brunner *et al*., 2005; Gravel *et al*., 2006). Joshi *et al.* (2010) also confirmed the growth enhancement activity of *Trichoderma* isolates.

The maximum growth enhancement in vanilla was observed in the *Trichoderma harzianum* and Pseudomonas *fluorescens* used followed by *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Pseudomonas putida*. *Trichoderma virens* showed the least effects and these results are in agreement with the previous reports such as the combinations of bacterial mixtures or bacteria and fungi, worked better than a single isolate (Anith *et al*., 2004). The combination *T. harzianum* (TR20) with *P. fluorescens* (P28) was most effective in reducing disease incidence (66.7% more effective than control) of seedling rot of chilly caused by *R. solani* and recorded high per plant yield (Rini and Sulochana, 2007). These strains could be of potential to develop as biofertilizers with biocontrol potential after testing their performance under field conditions either alone or as components of integrated disease nutrient management systems.

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