

# Effect of endophytic bacteria *Bacillus subtilis* EPC 5 on basal stem rot disease spread and nut yield in coconut plantations

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## Abstract

The wood degrading fungi *Ganoderma lucidum* (Leys) Karst causing Basal Stem Rot (BSR) in coconut plantation is the most destructive disease in Southern India. The control of this soil borne pathogen is particularly complex due to their occurrence in dynamic environment at the interface of the root with the soil. Hence, combinations of biocontrol agents differing in their mode of action especially with endophyte have been explored. The endophytic bacteria *Bacillus subtilis* EPC 5 from healthy coconut palm root showed high inhibition activity among fifty five isolated strains. Further, talc-based formulation incorporating *Pseudomonas fluorescens* Pf1 and *Trichoderma viride* Tv1 were developed and used individually, in combination to manage highly virulent, laccase producing soil borne pathogen. The result indicated that soil application of above mentioned antagonist in combination @ 100 g each enriched with organic substrate for four times per year, significantly reduced the disease severity with high nut yield at two different locations of disease endemic areas.

**Key words:** Coconut; basal stem rot (BSR), *Ganoderma*, endophytic bacteria and bioconsortia

**Abbreviations:** EPC – endophytes coconut; BSR- basal stem rot

## Introduction

Basal Stem Rot (BSR) disease caused by *Ganoderma lucidum* (Leys) Karst. is the most destructive and a major limiting factor in coconut production due to its soil borne nature and high laccase enzymes (Rajendran *et al.*, 2008). It is also called as Thanjavur wilt, bole rot, *Ganoderma* disease and Anabe roga in different states of India and occurs in numerous perennial, coniferous and palmaceous hosts. It is serious in sandy or sandy loam soils of coastal areas and easily spread to nearby palms by flood irrigation normally practiced by the farmers. Hence, management practices were found effective only when the disease was detected in the early stages (Bhaskaran *et al.*, 1996; Bejo and Vong 2014) and by use of integrated approach. Currently, no cost-effective fungicide that gives guaranteed control exists, although the disease could be delayed by adopting strategic management, which is a labour-intensive procedure. To date, no satisfactory method exists to control BSR under field condition. Growing concerns about environment and the high cost of chemicals have encouraged researchers to find out alternatives such as the use of biocontrol agents which has been reported by many workers (Susanto *et al.*, 2005; Naher *et al.*, 2013). Among bioagents, endophytes which reside internally in the plant tissues without causing visible harm to their hosts can be used in disease management (Wulff, 2000). The antagonists which differed in their ecology could be combined so that they could effectively utilise the root exudates and survive in association. Application of single biocontrol agent can may have limitations with regard to consistency and efficacy in different environments (Gumede, 2008). One of the important strategies to overcome these limitations is combining two or more beneficial microorganisms in a single application, in which they can adapt to different environmental circumstances which may complement each other (Atef 2008; Kildea *et al.*

2008). Domenech *et al.* (2006) reported that the combination of LS213 [product formed by *B. subtilis* strain GB03 (a growth-promoting agent), *B. amyloliquefaciens* strain IN937a (an inducer of systemic resistance) and chitosan] and *P. fluorescens* CECT 5398 increased all parameters (caliper, fresh weight and height) achieving the highest values in tomato and pepper. Incorporation of composted cow dung manure (5 %, vol/vol) into the light peat mix significantly decreased Botrytis Blight of Begonia severity while shoot dry weight and salability were increased (Horst *et al.*, 2005). Vermicompost-based bioformulations of *Pseudomonas fluorescens* Pf1 in combination with either *Trichoderma asperellum* TTH1 or *B. subtilis* EPCO-16 performed better in reducing sugarbeet root rot (Thilagavathy *et al.*, 2013). The soil application of bioconsortia enriched with farm yard manure (FYM) enhanced the coconut saplings growth under greenhouse conditions and showed higher induction of defense related enzymes like peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols when challenged with pathogen (Rajendran *et al.*, 2015).

With the background information, the present study was undertaken at the Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, India to investigate the effect of endophytic bacteria *B. subtilis* EPC5, *P. fluorescens* Pf1 (inducer of systemic resistance) and *T. viride* (high chitinase producer) individually or in combination enriched with organic substrate FYM for the management of basal stem rot disease which causes huge economic loss to the coconut plantations under endemic field conditions. The hypothesis tested was: Biocontrol agents in combination in higher dose reduce the *Ganoderma* infection and promotes higher nut yield.

## Materials and methods

**Pathogen and bioagents:** The *Ganoderma* pathogen was isolated from disease endemic areas and showed faster mycelial growth and higher laccase activity under *in vitro* conditions (Rajendran *et al.*, 2008).

**Endophytic bacteria isolation:** Coconut root samples were taken and brought to the laboratory. Root sections (2-3 cm long) were made using a sterile scalpel. Root samples were surface sterilized with 1 % sodium hypochlorite (NaOCl) in 0.05 % triton X-100 for 10 min. and rinsed four times in 0.02 M sterile potassium phosphate buffer pH 7.0 (PB). A 0.1 mL aliquot was taken from the final buffer wash and transferred to 9.9 mL tryptic soy broth (TSB) to serve as sterility check. Samples were discarded if growth was detected in the sterility check samples (agitating samples in TSB, Hi Media Code No. M 011, at 28±2 °C) within 48 h. Each sample (0.5 g) was triturated with a sterile mortar and pestle in 9.5 mL of the final buffer wash. Serial dilutions (up to 10<sup>10</sup>) of the triturate were made in phosphate buffer. Each dilution of every sample was plated (0.1 mL) on three plates each of three different media; Tryptic soy agar (TSA- Hi Media, Code No. M290), nutrient agar (NA g/l; peptone 5, beef extract 2 and agar 20, pH 5.0) and King's B Medium (g/L; proteose peptone 20, K<sub>2</sub>HPO<sub>4</sub> 1.5, Mg SO<sub>4</sub> · 7H<sub>2</sub>O 1.5, glycerol 20 mL and agar 15, pH 7.2). The plates were incubated at 28±2°C for 48-72 h. At each sampling date and for each treatment, one representative of each bacterium, as evident from their colony type and morphology was transferred to fresh King's B Medium plates to establish pure cultures.

**Talc-based formulation of strain and mixtures:** A loopful of bacterium was inoculated into the King's broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28±2°C). After 48 h of incubation, the broth containing 9 × 10<sup>8</sup> cfu/mL was used for the preparation of talc-based formulation. To the 400 mL of bacterial suspension, 1 kg of the purified talc powder (sterilized at 105 °C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, following the method described by Vidhyasekaran and Muthamilan (1995). After an overnight shade drying, it was packed in polypropylene bag and sealed. The bacterial strains, *Pseudomonas*, *Bacillus*, and fungal strain *T. viride* were grown separately in respective broth.

**Enrichment with organic substrate:** The organic substrate *viz.*, dried cow dung was tested for the multiplication of *Pseudomonas*, *Bacillus* and *T. viride*. One hundred gram of substrate was weighed in polypropylene bag, moisture content was adjusted to 60 % (W/V) by gravimetric method (Dutta and Das, 1999). One set of treatments of the above substrate were sterilized and another set was kept without sterilization. For sterilization the substrates were autoclaved at 15 lb psi for 1h on two consecutive days. The substrate was inoculated with 5 g of talc based bioformulation of *Pseudomonas*, *Bacillus*, *T. viride* and were incubated at room temperature. One gram of sample from FYM was derived at regular intervals, and the population (CFU) of *Pseudomonas*, *Bacillus* and *T. viride* in substrate and talc were counted at 10, 20, 30, 40, 50, 60 days intervals after inoculation by serial dilution techniques in selective medium for each bioagents.

**Field experiments:** The field experiments were carried out in two different BSR endemic locations of Tamil Nadu with 17 treatments and the treatments were replicated twice at three palms per replication to test the efficacy of endophytic consortia bioformulations enriched with FYM against basal stem rot disease. A randomized block design was used in the experiments. Production practices were followed as recommended by Tamil Nadu Agricultural University, Coimbatore, India. Disease index for each tree was calculated initially. In the field trials, all the bioagent treatments except chemical treatment were mixed with FYM, heaped for 15 days with regular watering, proper turning and applied 5kg to individual coconut tree. The different treatments are as follows.

1. Pfl (P) 150 g enriched with 5 kg FYM
2. EPC 5 (B) 150 g enriched with 5 kg FYM
3. Tv (T) 150 g enriched with 5 kg FYM
4. P + B 150 g enriched with 5 kg FYM
5. P + T 150 g enriched with 5 kg FYM
6. B + T 150 g enriched with 5 kg FYM
7. P + B + T 150 g enriched with 5 kg FYM
8. P 300 g enriched with 5 kg FYM
9. B 300 g enriched with 5 kg FYM
10. Tv 300 g enriched with 5 kg FYM
11. P + B 300 g enriched with 5 kg FYM
12. P + T 300 g enriched with 5 kg FYM
13. B + T 300 g enriched with 5 kg FYM
14. P + B + T 300 g enriched with 5 kg FYM
15. FYM 5kg
16. Tridemorph 2/100mL
17. Control

The bioagents applied at three months interval (four doses per year). The BSR disease intensity was assessed as disease index with the formula

$$\text{Disease index} = 23.6 + 17.7h + 3.6r - 0.6l ;$$

Where, h – Height of bleeding patches from ground level in metres, l – Number of functional leaves, r – Reduction in leaf size based on 0 – 4 scale. The initial disease index was recorded before imposing the treatments. Then the disease index was recorded periodically after imposing the treatments. Regular observations were made on the bleeding height and number of functional leaves.

**Nut yield:** The nut yield was recorded at the time of harvest for all the treatments.

**Statistical Analysis:** The data were statistically analyzed (Rangasamy, 1995) using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines. The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at significant level ( $P < 0.05$ ) and means were compared by Duncan's Multiple Range Test (DMRT).

## Results

**Endophytes:** Endophytic bacterial isolates EPC5, EPC8, EPC12, EPC13, EPC15, EPC21, EPC29, EPC32, EPC52 and *P. fluorescens* strain Pfl were found to increase the vigour index significantly. No external symptoms appeared after endophytic bacteria treatment. The strain, Pfl showed high inhibition to

*G. lucidum* followed by EPC5 (coconut root isolate) and EPC8 (coconut root isolate). The per cent inhibition was significantly higher in plates streaked with Pf1 (40.67 %), EPC5 (33.80 %) and EPC8 (29.16 %) against control plates. The bioagents EPC5, Pf1 and *T. viride* were tested in combination against *G. lucidum* *in vitro*. These three bioagents effectively inhibited the growth of pathogen even upto one month whereas in control, *G. lucidum* covered the plate within 6 days after placing the disc. Cloning and sequencing of 16s rDNA of EPC 5 belong to *B. subtilis* (Accession No. EF139862)

**Talc-based formulation of strain and mixtures:** At the time of application, the population of bacteria in talc formulation was  $2.5$  to  $3 \times 10^8$  cfu/g. Talc-based formulation prepared for each bioagents and mixed at the time of application after enrichment with the organic substrate.

**Enrichment with FYM:** To test the population buildup of bioagents *in vitro*, the effective bioagent formulations were mixed with sterilized and unsterilized FYM separately and in combination and heaped for different days. The population of bioagents was assessed at different days interval *viz.*, 10, 20, 30, 40, 50 and 60 days after mixing with FYM. The results showed that twenty days incubation was optimum for the maximum population buildup of *Bacillus*, *T. viride* and *Pseudomonas* in

both sterilized and unsterilized FYM (Fig 1).

**Bioconsortia under field conditions:** Soil application of these treatments around the basin of coconut palm and fungicide application by root feeding resulted in arresting the *G. lucidum* pathogen in both the field trials. The initial disease index was calculated for all the trees before imposing the treatments. After four doses of applications, results showed that EBC5+Pf1+Tv @ 300 g (100 g each) per palm enriched with 5 kg FYM effectively arrested the bleeding symptom in coconut palms compared to individual application. Results of the field experiments from trial I revealed that the palms treated with the mixture of bioformulation EPC5+Pf1+Tv @ 300 g (100 g each) per palm enriched with 5kg FYM as soil application recorded the disease index of 12.02 as against the disease index of 23.32 in untreated control palms, respectively (Table 1). Similar trend of results were obtained from field trial II, EPC5+Pf1+Tv @ 300 g/palm enriched with 5 kg FYM treatment recorded the basal stem rot disease index of 8.87 as against 25.86 in control. The standard check, root feeding of Tridemorph 2 % @ 100 mL/palm recorded the basal stem rot disease index of 11.18 (Table 1). Thus, the combination of different mixture of bioagents was found to be effective in reducing *G. lucidum* than the individual bioagents.

**Nut yield:** The results revealed that there was no significant difference between the treatments and there was significant

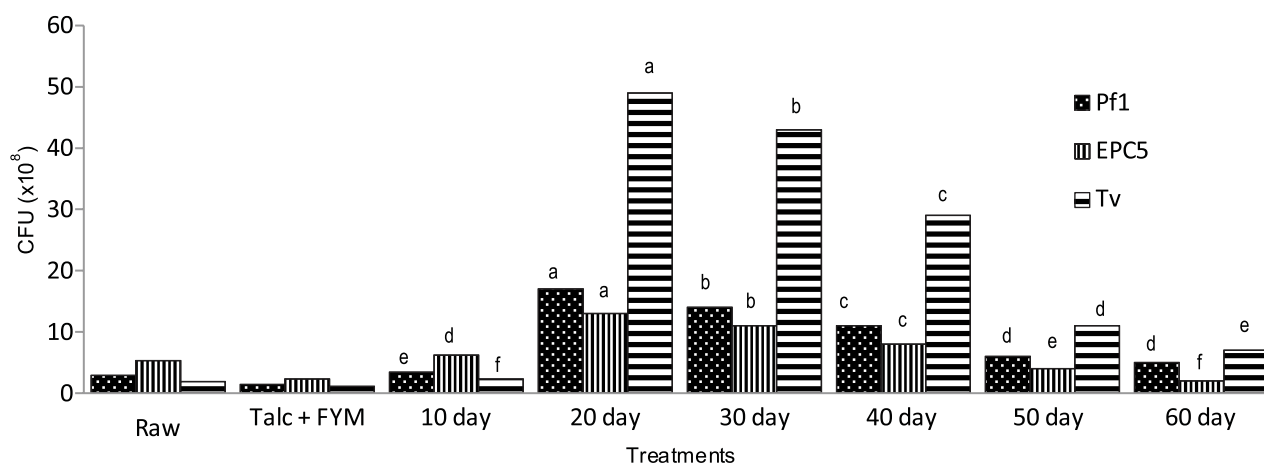


Fig 1. Population load of bioagents in FYM and Soil application in the coconut plantations

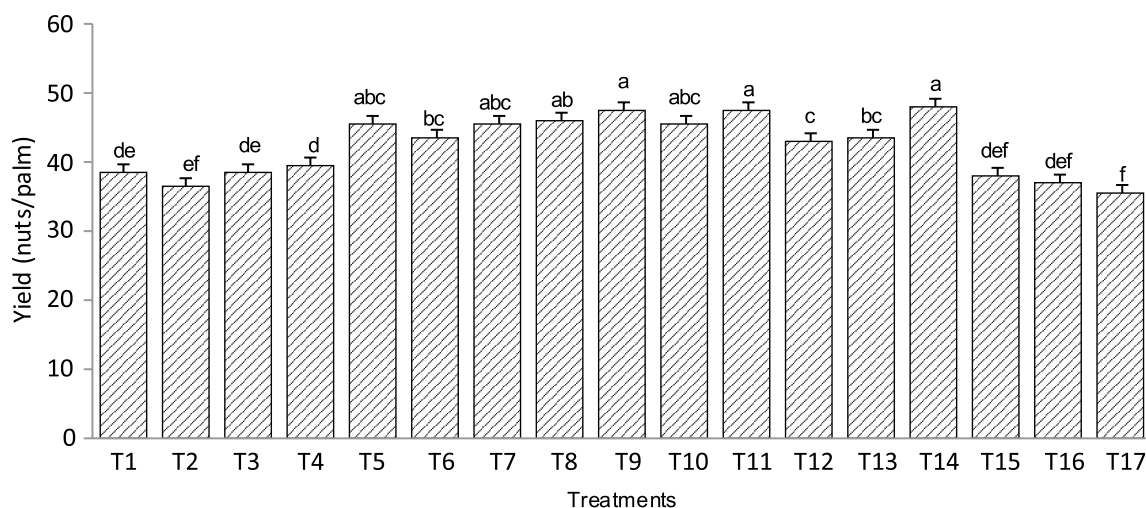


Fig 2. Nut yield in field trial I. Each value is the mean of two replicates of three harvest data. The values with the same letter in a column are not significantly different from each other according to Duncan's multiple range test at  $P = 0.05$

Table 1. Effect of biocontrol agents on the severity of basal stem rot disease of coconut

Treatments	Trial – I Disease index *		Trial – II Disease index *	
	Before Application	Final dose after application	Before Application	Final dose after application
P 150g + 5 kg FYM	9.143	11.200 <sup>f</sup>	10.493	11.985 <sup>h</sup>
B 150g + 5 kg FYM	3.339	5.500 <sup>ab</sup>	7.288	8.875 <sup>c</sup>
T 150g + 5 kg FYM	4.977	6.495 <sup>bc</sup>	13.883	15.565 <sup>k</sup>
P + B 150g + 5 kg FYM	4.018	5.295 <sup>ab</sup>	12.047	13.290 <sup>i</sup>
P + T 150g + 5 kg FYM	6.476	7.730 <sup>cd</sup>	7.483	8.835 <sup>d</sup>
B + T 150g + 5 kg FYM	3.385	4.660 <sup>a</sup>	9.385	10.665 <sup>g</sup>
P + B + T 150g + 5 kg FYM	3.078	4.270 <sup>a</sup>	6.986	8.185 <sup>cd</sup>
P 300g + 5 kg FYM	3.698	4.840 <sup>a</sup>	7.109	8.145 <sup>c</sup>
B 300g + 5 kg FYM	6.796	7.988 <sup>cde</sup>	5.900	7.235 <sup>b</sup>
T 300g + 5 kg FYM	3.189	4.460 <sup>a</sup>	4.422	5.260 <sup>a</sup>
P + B 300g + 5 kg FYM	3.293	4.825 <sup>a</sup>	8.815	9.665 <sup>f</sup>
P + T 300g + 5 kg FYM	7.345	8.675 <sup>de</sup>	6.800	7.890 <sup>bc</sup>
B + T 300g + 5 kg FYM	6.293	7.580 <sup>cd</sup>	13.389	14.220 <sup>j</sup>
P + B + T 300g + 5 kg FYM	10.945	12.015 <sup>f</sup>	8.191	8.870 <sup>e</sup>
FYM 5kg	6.270	9.380 <sup>c</sup>	13.530	20.070 <sup>l</sup>
Tridemorph 2/100ml	5.955	7.020 <sup>c</sup>	10.589	11.180 <sup>g</sup>
Control	10.978	23.320 <sup>g</sup>	13.075	25.855 <sup>m</sup>
P=0.05		1.647		0.681

\* Mean of two replications, Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at  $P = 0.05$ , P – *P. fluorescens* (Pf1), B – *B. subtilis* (EPC5), T – *T. viride* (Tv).

difference with control palms. Soil application of EPC5+Pf1+Tv @ 300 per palm along with 5 kg FYM recorded a higher number of nuts (48 nuts/palm) compared to control (35.5 nuts/palm) in field trial I at Dharapuram (Fig 2). Similar trend of results were obtained from field trial II at Pattukkottai, where the mixture of bioagents recorded 63.5 nuts/palm as against 52.5 nuts/palm in control (Fig. 3).

## Discussion

Coconut palms are affected by many numbers of pests and diseases. Among them, basal stem rot (BSR) disease caused by *Ganoderma lucidum* is the most destructive one which is widespread in nature. The term endophyte refers to microorganism that colonise intercellular spaces and vascular tissues of plants

without exhibiting pathogenicity and are believed to originate from the rhizosphere or phylloplane microflora. Introducing endophytic bacteria to the roots to control plant disease involves manipulation of the indigenous bacterial communities of the roots in a manner, which leads to enhanced suppression of soil-borne pathogens. The use of endophytic bacteria should thus be preferred to other biological control agents as they are internal colonizers, with better ability to compete within the vascular systems, limiting *Ganoderma* for both nutrients and space during its proliferation. This study was therefore, undertaken to isolate endophytic bacteria from symptomless coconut palm roots for the management of basal stem rot disease as bioconsortia. Endophytes are found in numerous plant species with the most being members of common soil bacterial genera such as *Pseudomonas*, *Bacillus* and *Azospirillum* (Chanway, 1998). Primed plants display either

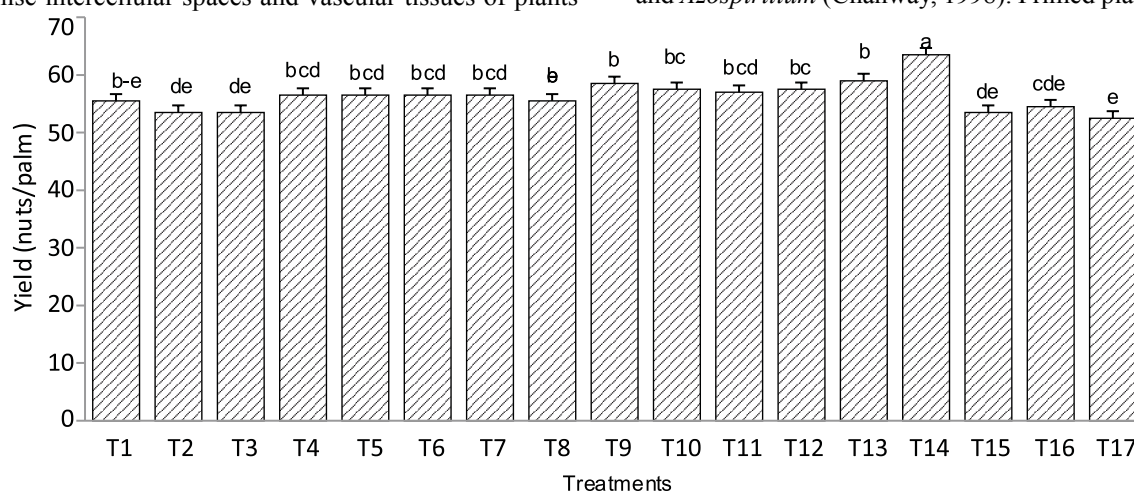


Fig. 3. Nut yield in field trial II. Each value is the mean of two replicates of three harvest data. The values with the same letter in a column are not significantly different from each other according to Duncan's multiple range test at  $P = 0.05$

faster, stronger or both activation of the various cellular defense responses that are induced following attack by either pathogens or insects or in response to abiotic stress (Conrath *et al.*, 2006). In the present study, endophytic bacteria from the roots of coconut palms EPC5, EPC8, EPC15, EPC29 and EPC52 were found to increase the vigour index of seedlings significantly when compared to untreated control. Most of the isolates were characterized as *Bacillus* spp. whereas some *Pseudomonas* spp. have also been identified. Similarly, Bivi *et al.* (2010) indicated that the bacterial endophytes from the genera *Pseudomonas*, *Burkholderia* and *Serratia* might have the potential to control *G. boninense*, as they were mostly found in healthy roots from symptomless palms. Several reports indicate the role of bacterial endophytes in the management of plant pathogens (Barka *et al.*, 2002). In the present study, EPC5 and Pf1 effectively inhibited the growth of *Ganoderma lucidum* *in vitro*. Thus an attempt was carried out in the present study to investigate the efficacy of some of the promising endophytic bacteria against basal stem rot pathogen. The endophytic bacteria have been reported to improve plant growth (Nejad and Johnson, 2000) and suppress the *Fusarium* spp., *Colletotrichum orbiculare*, *Rhizoctonia solani*, *Sclerotium rolfsii* (Pleban *et al.*, 1995). In the present study, Pf1 and endophytic bacterial strain EPC5 showed maximum mycelial inhibition of *G. lucidum*. Antagonistic strain, *Pseudomonas putida* (B0) isolated from a sub-alpine source exhibited antifungal activity against phytopathogenic fungi in Petri dish assays and produced chitinase,  $\beta$ -1, 3-glucanase, salicylic acid, siderophore and hydrogen cyanide (Pandey *et al.*, 2006). The inhibitory action may be due to the production of antifungal or antibacterial agents, antibiotics (Cazorla *et al.*, 2006), siderophore production and competition for nutrition. Seven strains of *Bacillus* isolates showed a high level of *in vitro* antagonism against *R. solani* and *Macrophomina phaseolina*. The above facts suggest that the inhibition of basal stem rot pathogen, *G. lucidum* by endophytic bacteria may be due to the production of antibiotics, siderophore mediated competition and lytic enzymes *viz.*, chitinase,  $\beta$  - 1, 3 glucanase. These antibiotics and lytic enzymes might have degraded the fungal cell wall and restricted the growth of fungus *in vitro*. Various phenotypic and molecular methods have been developed and used for characterizing bacterial isolates. The result obtained by analyzing primary character and carbon source utilization of different endophytic bacteria revealed that they belong to *Bacillus* spp. Tilak and Srinivasa Reddy (2006) identified isolates from maize as *Bacillus cereus* and *B. circulans* by biochemical characteristics and profile of fatty acids. *Trichoderma* strains are among the most studied fungal biocontrol agents and are successfully used as biopesticides and biofertilizers in greenhouse and field plant production (Harman *et al.*, 2004). The soil borne filamentous fungus *Trichoderma virens* is a biocontrol agent with a well known ability to produce antibiotics, parasitize pathogenic fungi and induced systemic resistance in plants. Pretreatment of cotton cotyledons with *Trichoderma* spp. provided high levels of protection to the foliar pathogen, *Colletotrichum* sp. and involved in the induction of resistance through the activation of plant defense mechanisms (Djonovic *et al.*, 2006). *Trichoderma* spp. are known to produce large quantities of fungistatic metabolites which are active against many soil borne plant pathogens. In our study, *T. viride* strongly inhibited the growth of *G. lucidum* *in vitro*. It was observed

that soil application of bioagents with different combinations promoted the growth in coconut seedlings significantly compared to untreated control under greenhouse conditions. There was a significant increase in the seedling height, girth, leaf area and number of leaves in the bioagents treated seedling. The result is supported by the findings of De Silva *et al.* (2000) the leaf area and stem diameter of high bush blueberry were increased by applying *Pseudomonas fluorescens* Pf5. Esitken *et al.* (2003) found that application of *Bacillus* strain OSU 142 as foliar spray at full bloom stage significantly increased the shoot length, yield and the nutrient element composition in apricot trees compared to control. The results revealed that the soil application of talc formulation of *Bacillus* + Pf1 + Tv @ 100g each/palm along with 5 kg FYM recorded significantly lower disease index over control after three applications. This observation supports the findings of Susanto *et al.* (2005) where they conducted glasshouse and field trial for *Ganoderma* control and indicated that treatment with *T. harzianum* and *Gliocladium viride* were superior to *Bacillus* sp. A large scale trial showed that the disease incidence was lower in oilpalm fields treated with biological agents than in untreated fields. In the present study, soil application of bioagents mixture resulted in enhanced nut yield of coconut in both the field trials. Field studies over a period of two years indicated that stem rot disease in canola was controlled by *Pseudomonas chlororaphis* and *Bacillus amyloliquefaciens* which were comparable with fungicide treatments (Fernando *et al.*, 2006).

To conclude, the strains *viz.*, EPC5, Pf1 and Tv in combination enriched with organic substrate performed well against basal stem rot disease in coconut palms. Future work requires an in depth analysis of the endophytes mediated BSR control, their optimum population density, as well as the best method of application.

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