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RESEARCH ARTICLE

Biopriming of micropropagated banana plants at pre- or post-BBTV inoculation stage with rhizosphere and endophytic bacteria determines their ability to induce systemic resistance against BBTV in cultivar Grand Naine

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ABSTRACT

Rhizospheric and endophytic bacteria isolated from the roots and corms of banana were tested to find out their efficiency in controlling against *banana bunchy top virus* (BBTV). Bioformulations of mixtures of endophytic *Bacillus pumilus* and *B. subtilis* isolated from banana cv. Grand Naine and rhizobacterial isolate *Pseudomonas fluorescens* (Pf1) were found to be effective in increasing the growth and physiological parameters such as pseudostem girth and height, number of leaves, phyllochron, and leaf area in biohardened plants under greenhouse study. The consortia of bioformulation mixture of *B. pumilus*, *B. subtilis*, and *P. fluorescens I* showed 61.62% disease reduction over control. The defence enzymes such as peroxidase (POX), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), and total phenol were induced to an elevated level in biohardened plants. The applications of bioformulations to plants led to delay the symptom expression for 63.75 to 70.50 days compared to control after challenge inoculation with the virus in 34–67% of plants that exhibited the symptoms till 150 DAI. However, biohardening of plants with the same combinations of bacteria three days after BBTV inoculation led to express the symptoms 29.16 to 36.71 days and there was a significant decrease in plant growth parameters. Biopriming prior to BBTV infection has attributed to the enhanced plant growth and resistance against BBTV whereas, the same treatments after virus inoculation did not induce resistance. This study has proved that the time of application of consortia of bio-inoculants determines their effect of induced resistance to BBTV in micropropagated plants.

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Endophytes; bioformulations; biocontrol; *banana bunchy top virus*; management

1. Introduction

Bananas and plantains (Musa sp.) are the major food crops cultivated in 10.3 million ha across 130 countries for meeting the need of the food security and livelihood of millions of people living in the tropical and subtropical regions of the world (Lava Kumar, Selvarajan, Iskra Caruana, Chabannes, & Hanna, 2015). Among various banana cultivars, cv. Grand Naine occupies more than 50% of the area under banana cultivation in India, and it is a

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widely accepted fruit for consumption and is also a crop that is exported around the world from India (Preethi & Balakrishna Murthy, 2013). Banana cultivation is delimited by many fungal, bacterial, and viral pathogens, and they cause a significant economic loss to the growers (Jones, 2000; Ploetz, 1998). Among the viral diseases, banana bunchy top disease (BBTD) caused by *banana bunchy top virus* (BBTV) is one of the major constraints in banana cultivation worldwide (Dale, 1987). In a survey conducted in 2008 in India, 17.16 million plants of Cavendish banana were found to be affected with BBTV in Jalgaon district of Maharastra and this caused an economic loss of around US$ 51 million (Selvarajan et al., 2010). BBTD causes significant yield losses in banana production due to lack of non-availability of control and management practices (Rishi, 2009). Except for timely eradication by roguing in early steps, no other control measures are available for managing the BBTV.

As an alternative to control approach using pesticides, biohardening is an emerging trend envisioned at reducing harmful chemical usage in plant production while enhancing plant fitness, productivity, and resistance to pests and diseases in the context of sustainable horticulture (Harish et al., 2007, 2008, 2009a, 2009b; Rajamanickam, Karthikeyan, Kavino, & Manoranjitham, 2018). A process of allowing the selected bacteria as endophytes in the plant root/corms either through root feeding or by soil drenching is called biohardening (Harish et al., 2008; Kumar et al., 2013). Among the banana endophytes, *Bacillus* spp. are frequently isolated from banana cv. Grand Naine (Thomas & Thyvalappil Soly, 2009). *Bacillus* spp. are widely exploited endophytes for induction of induced systemic resistance (ISR) against many plant pathogens (Chen et al., 2013; Choudhary & Bohri, 2009; Kloepper, Ryu, & Zhang, 2004) and for improving plant growth and yield parameters. In this context, we studied whether the application of rhizosphere and endophytic microbes to tissue culture banana plants will significantly increase the growth parameters by physiological changes, thereby leading to increased resistance to BBTV and also to know if the virus was inoculated to plants prior to application of bio-inoculants shall confer induced systemic resistance. Therefore, the present study was aimed to understand the utility of inoculation of endophytic bacteria, viz. *B. pumilus* (BP) and *B. subtilis* (BS) isolated from banana cv. Grand Naine and rhizosphere bacteria, *Pseudomonas fluorescens* (PfI), in tissue-cultured banana plants to obtain improved resistance against BBTV.

2. Material and methods

2.1. Source of plants

Ten daughter suckers from healthy vigorously growing banana clumps of cv. Grand Naine in Theni district of Tamil Nadu, India, were chosen to isolate endophytic bacteria. Since this area is known for the higher incidence of BBTV, we chose to find vigorous plants for the isolation of endophytes. In the case of pot culture experiments, 45-day-old, virus-free certified, hardened tissue culture plants of cv. Grande Nain were obtained from M/s Jain Irrigation Systems Limited, Jalgaon, India. The healthy tissue culture banana plants were tested for the presence of BBTV by PCR before they are used in pot culture experiments.

2.2. Isolation of endophytic bacteria and molecular characterisation

The endophytic bacteria were isolated from the internal tissues of the pseudostem and rhizome of banana cv. Grand Naine as described by Quadt-Hallmann, Hallmann, and

Kloepper (1997). The rhizobacterial isolate PfI was obtained from TNAU, Coimbatore, Tamilnadu, India. The standard roll towel method was used to calculate the vigour of the rice seedlings after the treatment with rhizospheric and endophytic bacteria (ISTA, 1993). The formula described by Abdul Baki and Anderson (1973) was used to calculate the vigour index induced by bacterial strains, and the best bacterial species were chosen for testing in banana tissue culture plants. Endophytic bacterial DNA was extracted as described by Robertson et al. (1999). Genotypic characterisation of isolated endophytes was carried out by 16s rDNA sequence amplification as described by Weisburg, Barns, Pelletier, and Lane (1991). The universal primers Eu27F 5′GAGAGTTTGATCCTGGCTCAG3′ and 1495R 5′CTAGGCTACTTGTTACGA3′ were used to amplify ~1520 bp targeted sequence in the 16s rDNA of the endophytic bacteria isolated in the study. The amplified products were cloned into pTZ57R/T vector (InsTAclone PCR cloning kit, Thermofisher Scientific, USA) according to the manufacturer's instructions and the recombinant clones were sequenced at Eurofin genomics India Pvt Ltd, Bangalore.

2.3. Preparation of bioformulation and biohardening of plants

The method described for preparing individual and mixtures of both rhizospheric and endophytic bioformulations was adopted from Nandakumar, Babu, Viswanathan, Raguchander, and Samiyappan (2001). Before biohardening, the tissue culture plants of cv. Grand Naine were indexed for the BBTV using PCR as described by Selvarajan et al. (2010) and the plants free of BBTV were used for the biohardening. The bacterial strains were grown separately and two or three strains that are going to make up the mixture were added equally (*v*/*v*) and finally mixed with talc powder, calcium carbonate, and carboxy methyl cellulose as described by Nandakumar et al. (2001). Root feeding was performed by a method described by Kumar et al. (2013) with slight modifications. For root feeding, the individual bacterial suspensions were prepared and two or three strains were mixed equally to prepare the required consortia, and the final concentration was $8.0–9.2 \times 10^8$ cfu/ml. Then, five roots per plant were washed with water and a fine cut was made at the tip of the roots and immersed in the selective bacterial suspensions (Figure 1) in sterile conditions for 30 min. The plants were planted in earthen pots (43 cm × 35 cm × 23 cm) having red soil, sand, farmyard manure at 1:1:1 ratio. After two months of root feeding, talc-based formulation of the endophytic bacterial strains at 10 g plant^{-1} (1%) was drenched in the pots (Harish, Kavino, Kumar, Balasubramanian, & Samiyappan, 2009a). Tissue culture plants drenched with water served as control. A pot culture experiment was conducted to find out the ISR against BBTV with applications of individual and different combinations of rhizospheric and endophytic bacteria *viz.* BP, BS, and PfI. The completely randomised design was used for the pot culture experiment with seven treatments replicated thrice with five plants per replication. The pot culture experiments laid to find out the effect of biohardening of tissue culture banana plants with rhizospheric and endophytic bacteria. In one set of experiment, BBTV was pre-inoculated before biohardening. Briefly, healthy virus-free tissue culture plantlets were inoculated with BBTV using ten viruliferous aphids. Later, the viral infection in plants was confirmed by the PCR amplification with BBTV CP gene primer. After three days, the virus pre-inoculated plants were treated with individual strains *viz.* BP, BS, and PfI and different combinations of bioformulations viz. BP + PfI, BS + PfI, and BP + BS + PfI and

Figure 1. Root feeding method of bacterial consortia for banana: The tissue culture banana plants of cv. Grand Naine roots were cut and kept in a sterile polypropylene box with the bacterial consortia for 30 min.

planted in pots as described previously and in another, BBTV was inoculated three months after the biohardening as per Harish et al. (2008). The total number of days taken to express the typical symptoms of BBTV and percent disease incidence were recorded for all the treatments based on Harish et al. (2008), and using these data, the percent disease reduction (PDR) over untreated control was calculated as described by Yogeeswarudu & Venkata Krishna (2014).

2.4. Aphid transmission and virus indexing

Three-month-old biohardened plants were inoculated with viruliferous aphids. Transmission of BBTV to tissue culture banana plants has been performed as described by Su, Tsao, Wu, and Hung (2003) with slight modifications. The BBTV-free banana aphids were transferred to the BBTV-infected leaf bits kept in a Petri dish-moist chamber for a period of 24 h to acquire the virus. Then, the viruliferous aphids were transferred to healthy tissue culture banana plants and allowed to feed for 48 h duration at 28 ± 2°C in closed insect chamber. After 48 h of transmission, the plants were sprayed with 0.02% imidacloprid. The virus-inoculated plants were kept in a glass house for observation. Leaf samples (100 mg) from every newly emerging leaf of inoculated plants were collected, and total DNA was isolated from leaf samples following the protocol of Selvarajan, Balasubramanian, Kavitha, Sathiamoorthy, and Ahlawat (2008) and the presence or absence of the BBTV was assayed using PCR as described by Selvarajan et al. (2010).

2.5. Influence of rhizospheric and endophytic bacteria on plant growth parameters

The plant growth parameters such as pseudostem girth and height, number of leaves, phyllochron (number of leaves produced per week), and total leaf area were recorded for all the potted tissue culture banana plants at different intervals. In the case of total leaf area, a formula described by Murray (1960) was used to estimate it. The formula followed was $TLA = L \times B \times K1 \times N$, wherein TLA is the total leaf area expressed in m^2: L, length; B, breadth; $K1$, a factor of 0.8; and N, number of leaves.

2.6. Total microbial load estimation

The pour plate technique was used to obtain the microbial count based on Cao et al. (2004). Briefly, 1 g of tissue was taken from the corm and pseudostem of each plant and surface sterilised by dipping the tissue in 3% sodium hypochlorite for 1 min followed by 70% ethanol for 1 min and finally washed three times with sterile distilled water. The success of surface sterilisation of tissues was ensured by streaking the aliquots of the distilled water used in the final rinse in nutrient agar plates, and no bacterial colonies were observed after 24 h. The samples were macerated in 3 ml of sterile distilled water using sterile mortar and pestle. Each sample was then serially diluted and plated. The plates were then incubated and observed for bacterial growth.

2.7. Assay of defence-related enzymes

The activity of plant defence enzyme peroxidase (POX) was assayed using a spectrophotometer as reported by Hammerschmidt, Nuckles, and Kuc (1982) and the enzyme, polyphenol oxidase (PPO), activity was assessed as described by Mayer, Harel, and Shaul (1965). The enzyme activity was expressed as the change in the absorbance of the reaction mixture/min/g on a fresh weight basis. The phenylalanine ammonia lyase (PAL) activity was calculated as described by Ross and Sederoff (1992), and it was the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. The total phenol content was expressed as catechol equivalents g^{-1} of protein after estimating it in banana leaf tissues by using the procedure described by Zieslin and Ben-Zaken (1993).

2.8. Statistical analyses

A completely randomised block design was used for the pot culture experiments. All data were analysed using Indian NARS Statistical Computing Portal. The data recorded on various characters were analysed using ANOVA. Means were compared by Duncans multiple range tests ($P \leq .05$).

3. Results

3.1. Effect of endophytic bacterial strains on plant growth promotion

Thirty-two endophytic bacteria were isolated from the internal tissues of banana plants collected from the orchards. Based on their vigour index on rice (Table 1), 16 endophytic bacterial species were chosen and identified by 16srDNA analysis (Figure 2). Most of the bacterial species were *Bacillus* spp., and based on the efficacy to induce root vigour index, *B. pumilus* and *B. subtilis* were chosen to assess their efficacy against the BBTV along with a rhizopheric PfI with different combinations.

3.2. Effect of biohardening on tissue culture plants of cv. Grand Naine under pot culture

The growth parameters in biohardened plants were recorded after three months of biohardening. There was a significant difference between treatments for all the parameters

Table 1. Screening of endophytic bacteria isolated from banana growth promotion in rice.

Endophytes	*G* (%)	Shoot length (cm)		Root length (cm)		Vigour index	
		RTM	PC	RTM	PC	RTM	PC
EPB1	31 ± 3.41^{ij}	3.62 ± 0.07^{hi}	$9.40 \pm 0.88^{b–g}$	2.61 ± 0.12^{kl}	5.40 ± 0.40^{mn}	$193.13^{l–o}$	458.8^{l}
EPB2	$49 \pm 5.50^{c–g}$	5.68 ± 0.12^{def}	$8.77 \pm 0.71^{b–g}$	$5.73 \pm 0.38^{f–j}$	3.99 ± 0.23^{n}	559.09^{de}	625.24^{ij}
EPB3	$37 \pm 6.80^{g–j}$	3.20 ± 0.12^{hi}	$9.99 \pm 0.94^{b–f}$	5.81 ± 0.82^{jkl}	$6.07 \pm 0.18^{j–m}$	333.37^{jk}	594.22^{ijk}
EPB4	65 ± 4.72^{b}	4.90 ± 0.81^{f}	$6.77 \pm 0.72^{b–g}$	$6.89 \pm 1.03^{h–k}$	$7.22 \pm 0.22^{k–l}$	766.35^{ab}	909.35^{bcd}
EPB5	$57 \pm 6.80^{b–e}$	4.60 ± 0.24^{fg}	4.37 ± 0.72^{gh}	$4.95 \pm 0.75^{e–h}$	2.85 ± 0.08^{n}	544.35^{def}	411.54^{lmn}
EPB6	26 ± 6.21^{j}	5.40 ± 0.12^{def}	$7.02 \pm 1.08^{b–f}$	7.32 ± 0.79^{bcd}	$7.22 \pm 0.31^{e–i}$	330.72^{jk}	$370.24^{l–o}$
EPB7	$48 \pm 4.32^{d–h}$	3.40 ± 0.12^{hi}	$6.77 \pm 0.42^{b–g}$	$4.10 \pm 0.86^{g–j}$	3.22 ± 0.67^{n}	360.00^{l}	479.52^{l}
EPB8	34 ± 4.16^{ij}	0.00^{k}	$4.75 \pm 0.63^{f–h}$	0.00^{m}	4.95 ± 0.25^{lm}	0.00^{p}	$329.8^{l–o}$
EPB9	54 ± 3.82^{bcde}	2.40 ± 0.20^{i}	$5.75 \pm 0.39^{e–h}$	1.85 ± 0.10^{l}	$6.22 \pm 0.47^{g–l}$	229.5^{lmn}	646.38^{ij}
EPB10	54 ± 2.58^{bcde}	5.20 ± 0.10^{ef}	$7.65 \pm 1.21^{a–e}$	$3.27 \pm 0.61^{i–l}$	9.27 ± 0.27^{abc}	457.38^{gh}	913.68^{bcd}
EPB11	27 ± 1.91^{ij}	5.50 ± 0.25^{def}	$5.95 \pm 0.29^{d–h}$	$4.77 \pm 0.48^{f–j}$	4.82 ± 0.19^{lm}	277.29^{m}	290.79^{p}
EPB12	56 ± 1.63^{bcd}	7.90 ± 0.40^{a}	$8.55 \pm 0.61^{a–d}$	$5.95 \pm 0.37^{c–f}$	$7.65 \pm 0.13c^{–h}$	775.6^{b}	907.2^{e}
EPB13	54 ± 3.82^{bcde}	2.90 ± 0.37^{hi}	$5.57 \pm 0.84^{e–h}$	2.32 ± 0.08^{kl}	$5.60 \pm 0.27i^{–m}$	281.88^{m}	603.18^{ij}
EPB14	25 ± 2.51^{j}	1.30 ± 0.21^{j}	3.87 ± 0.72^{h}	1.62 ± 0.34^{l}	$7.40 \pm 0.26^{d–h}$	$73^{l–o}$	281.75^{p}
EPB15	58 ± 2.58^{bcd}	7.30 ± 0.43^{abc}	$6.77 \pm 0.55^{b–g}$	7.87 ± 0.69^{b}	$7.95 \pm 0.93^{b–g}$	879.86^{ab}	853.76^{efg}
EPB16	79 ± 3.00^{a}	4.60 ± 0.28^{fg}	$8.50 \pm 1.27^{a–d}$	$5.15 \pm 0.59^{e–h}$	$6.27 \pm 0.53^{g–l}$	770.25^{ab}	1166.83^{a}
EPB17	62 ± 2.58^{bcd}	5.80 ± 0.49^{def}	$7.62 \pm 0.54^{a–e}$	$5.87 \pm 0.68^{c–f}$	$6.77 \pm 0.74^{f–k}$	723.54^{ab}	892.18^{ef}
EPB18	63 ± 3.41^{bcd}	7.80 ± 0.28^{a}	$7.67 \pm 0.76^{a–e}$	7.00 ± 0.31^{bcd}	$8.22 \pm 1.23^{b–f}$	932.4^{a}	1001.07^{bc}
EPB19	27 ± 4.43^{ij}	0.00^{k}	$7.07 \pm 0.55^{b–f}$	0.00^{m}	5.37 ± 0.27^{klm}	0.00^{p}	335.88^{lmn}
EPB20	$38 \pm 6.21^{f–j}$	3.70 ± 0.17^{gh}	0.00^{i}	10.70 ± 0.38^{a}	0.00^{o}	547.2^{def}	0.00^{q}
EPB21	81 ± 3.41^{a}	6.52 ± 1.11^{bcd}	$8.57 \pm 1.50^{a–d}$	$6.15 \pm 0.61^{c–f}$	9.40 ± 0.30^{ab}	1026.27^{a}	1455.57^{a}
EPB22	58 ± 2.58^{bcd}	5.37 ± 0.51^{def}	8.65 ± 0.26^{abc}	$4.75 \pm 0.62^{f–j}$	$6.85 \pm 0.84^{f–k}$	586.96^{d}	899^{ef}
EPB23	$51 \pm 5.74^{b–f}$	6.57 ± 0.42^{bcd}	$8.12 \pm 0.46^{a–e}$	$5.72 \pm 0.70^{d–g}$	10.57 ± 0.35^{a}	626.79^{c}	953.19^{bcd}
EPB24	64 ± 3.65^{bc}	7.65 ± 0.27^{ab}	$8.55 \pm 1.46^{a–d}$	$5.82 \pm 0.21^{c–f}$	$7.72 \pm 0.37^{b–g}$	862.08^{ab}	1038.08^{b}
EPB25	60 ± 4.32^{bcd}	3.30 ± 0.51^{hi}	$6.75 \pm 0.73^{b–g}$	$5.95 \pm 0.48^{c–f}$	$7.15 \pm 0.22^{e–j}$	555^{de}	$834e^{fg}$
EPB26	35 ± 3.41^{hij}	5.57 ± 0.45^{def}	$6.32 \pm 0.65^{c–g}$	$3.60 \pm 0.22^{h–k}$	$7.57 \pm 0.12^{c–h}$	320.95^{jkl}	486.15^{ijk}
EPB27	60 ± 7.11^{bcd}	4.97 ± 0.69^{ef}	$6.55 \pm 0.33^{b–g}$	1.85 ± 0.22^{l}	$7.02 \pm 1.29^{e–k}$	409.2^{ghi}	$814.2^{e–h}$
EPB28	55 ± 5.25^{bcde}	0.00^{k}	$7.57 \pm 0.39^{a–e}$	0.00^{m}	$6.25 \pm 0.29^{g–l}$	0.00^{p}	760.1^{i}
EPB29	58 ± 2.58^{bcd}	6.20 ± 0.49^{cde}	$8.05 \pm 0.46^{a–e}$	8.20 ± 0.66^{b}	$8.22 \pm 0.71^{b–f}$	835.2^{ab}	943.66^{bcd}
EPB30	60 ± 6.32^{bcd}	5.50 ± 0.47^{def}	$6.65 \pm 0.35^{b–g}$	7.45 ± 0.23^{bc}	$5.90 \pm 0.36^{h–l}$	777^{b}	753^{i}
EPB31	$41 \pm 1.91^{e–i}$	5.65 ± 1.05^{def}	9.12 ± 0.46^{ab}	$6.57 \pm 0.32^{b–e}$	$9.00 \pm 0.43^{a–d}$	501.02^{g}	742.92^{i}
EPB32	58 ± 4.16^{bcd}	0.00^{k}	10.10 ± 0.55^{a}	0.00^{m}	$8.67 \pm 0.41^{b–e}$	0.00^{p}	1088.66^{b}

Note: Vigour index = germination% × seedling length. Each value represents the mean of four replicates of 25 seeds each (±SE) after 15 days. RTM, roll towel method; PC, pot culture. Means with the same letter are not significantly different from each other at $\alpha = 0.05$ according to DMRT test.

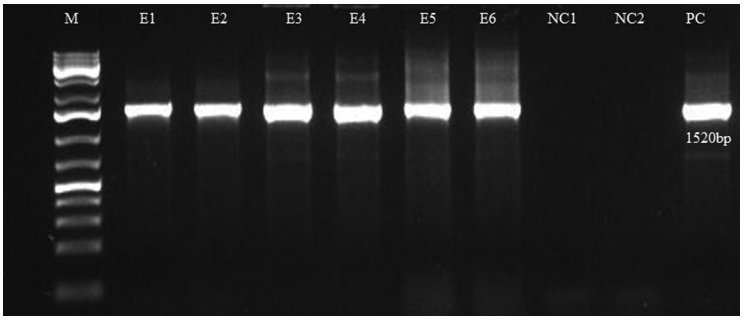

Figure 2. PCR amplification of endophytic bacterial 16srDNA gene. Lanes: M – 1 kb marker DNA; E1–E6, 16srDNA amplicon of endophytic bacterial DNA; NC1–NC2, negative control; PC, positive control.

recorded, viz. pseudostem height and girth, number of leaves, phyllochron, and the total leaf area (Table 2). Among the different treatments, T6, the mixture or consortia of all three bacteria (PfI + BS + BP) exhibited the maximum height closely followed by T4 (Pf1 + BP) and the least was recorded in the control (Figure 3). The girth was maximum in BP + Pf1-treated plants followed by BP-alone-treated plants. Plants treated with BP alone, BP + Pf1 and consortia of all three (T6) produced a maximum number of leaves and were on par with each other. Significant differences were observed among the treatments for phyllochron in different endophytic bacteria-treated tissue culture banana plants. The consortia of three bacteria (T6) and T5, a mixture of BS + PfI recorded the faster rate of leaf production followed by T1 and T4. The phyllochron was least in control plants. Among the treatments, T6, a mixture of three bacteria (BP + BS + PfI) and T5 (BS + Pf1) were on par and superior in registering maximum total leaf area, which is followed by BP + PfI.

3.3. Assay of defence enzymes

The defence enzymes such as POX, PPO, and PAL were assayed after three months after planting. The PAL activity was highest in BP + BS + PfI-combined-treated plants (Table 3)

Table 2. Morphological characters for the biohardened plants measured three months after planting in pots.

Treatment	Height (cm)	Girth (cm)	No. of leaves (*N*)	Phyllochron	Leaf area (cm^2)
T1	28.75^{e}	8.42^{b}	8.83^{a}	5.75^{b}	3019.10^{d}
T2	31.00^{d}	7.75^{d}	7.58^{c}	6.08^{c}	3100.32^{c}
T3	31.58^{d}	7.83^{d}	7.42^{c}	6.00^{c}	3153.60^{c}
T4	35.33^{b}	8.92^{a}	8.42^{a}	5.92^{b}	3581.33^{b}
T5	33.08^{c}	8.00^{c}	7.75^{b}	5.58^{a}	3603.07^{a}
T6	36.00^{a}	8.08^{c}	8.67^{a}	5.58^{a}	3609.33^{a}
Control	23.83^{f}	7.08^{e}	6.92^{d}	6.75^{d}	1729.45^{e}
General mean	31.37	8.01	7.94	5.95	3113.74
P value	<.0001	0.0002	<.0001	0.0008	<.0001
F value	1.5827	0.8675	0.7833	0.2568	0.7833
Significant	**	**	**	**	**

Note: Values are the means of three replicates. Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $P = .05$. T1, *B. pumilus* (BP); T2, *B. subtilis* (BS); T3, *Pseudomonas fluorescens* I (Pfl); T4, BP + Pfl; T5, BS + Pfl; T6, BP + BS + Pfl; Control, water-treated plants.
*Significant at 5%; **Significant at 1%; NS, non-significant.

Figure 3. A view of biohardened tissue culture banana plants with higher pseudostem girth, height, leaf area and more number of leaves compared to control (left).

followed by T4 (BP + PfI-treated plants). In case of POX, T4 recorded to be highest followed by T6, T5, and T1. PPO activity was maximum and equal in T6 and T5. Total phenol content was not significant among the treatments. Overall, a significant increase in all the three plant defence enzymes assayed in this study was noticed in biohardened plants compared to control.

3.4. Total microbial load

The total microbial load was assessed three months after biohardening. The total microbial load was significantly higher in all biohardened plants compared to non-treated control. The highest microbial load (7.4×10^7 cfu/g) was recorded in T6, i.e. plants treated with consortia of BP + BS + PfI followed by T3, T5, and T4 (Figure 4). However, in control, the microbial load was least (2.9×10^7 cfu/g).

3.5. Effect of biohardening in micro propagated banana against BBTD in pot culture

3.5.1. Inoculation of BBTV prior to biohardening

In the first experiment in which the BBTV was pre-inoculated prior to biohardening, the percent infection in these pre-inoculated plants ranged from 66% to 80%, whereas in control the PI was 86%. The PI of different treatments clearly showed that there was no influence of biohardening with rhizospheric and endophytic bacteria. In all the treatments, the plants expressed typical BBTV symptoms within 29.16–36.71 days after inoculation. BS + PfI-treated plants showed a maximum delay in symptom expression with 36.71 days. The consortia of BP + BS + PfI-combined-treated plants expressed symptom within 29.16 days. The PDR over control ranged from 6.97 to 23.25. BS-treated plants showed a maximum of 23.25%PDR over control followed by 15.11% in T5 (BS + Pf1-treated) (Table 4). We have tested all the treated plants through PCR at regular intervals. Fourteen per cent of the non-symptomatic biohardened plants from the virus pre- or post-inoculated showed PCR positive up to 100 DAI (Figure 5). The data on plant growth parameters, viz., height, girth, number of leaves, phyllocron and leaf area of all the

Table 3. Assay of defence enzyme for the biohardened plants measured three months after planting pots.

Treatment	Peroxidase	Polyphenol oxidase	PAL	Phenol
T1	0.0025^{b}	0.0034^{c}	3.06^{c}	0.05^{c}
T2	0.0019^{d}	0.0040^{b}	1.33^{e}	0.12^{a}
T3	0.0022^{c}	0.0042^{b}	1.77^{e}	0.06^{b}
T4	0.0031^{a}	0.0036^{c}	4.64^{b}	0.05^{c}
T5	0.0026^{b}	0.0048^{a}	3.27^{d}	0.06^{b}
T6	0.0028^{b}	0.0048^{a}	5.48^{a}	0.03^{d}
Control	0.0019^{d}	0.0019^{d}	1.24^{f}	0.02^{e}
General mean	0.0024	0.0028	2.97	0.06
P value	<.0001	<.0001	0.0050	0.1751
F value	12.4557	8.2711	3.4093	1.9221
Significant	**	**	**	NS

Note: Values are the means of three replicates. Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $P = .05$. PO: changes in absorbance at 470 nm min^{-1} g^{-1} fw. PPO: changes in absorbance at 470 nm min^{-1} g^{-1} fw. PAL: nmol of trans-cinnamic acid min^{-1} g^{-1} fw. Phenol: catechol equivalents g^{-1} fw. T1, *B. pumilus* (BP); T2, *B. subtilis* (BS); T3, *Pseudomonas fluorescens* I (Pfl); T4, BP + Pfl; T5, BS + Pfl; T6, BP + BS + Pfl; Control, water-treated plants

*Significant at 5%; **Significant at 1%, NS, non-significant.

biohardened plants pre-inoculated with BBTV, revealed a significant reduction in all the growth parameters recorded three months after inoculation compared to un-inoculated healthy plants (Table 5). The virus-inoculated control without biohardening also showed a significant reduction in all growth parameters recorded compared to un-

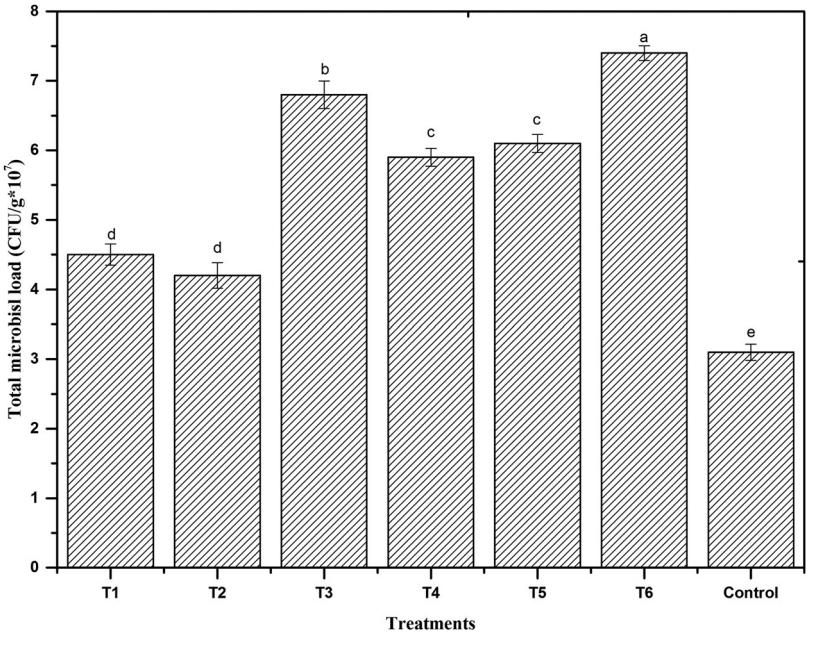

Figure 4. Estimation of total microbial load in biohardened plants. *Means are the average of three replicates. T1, *B. pumilus*; T2, *B. subtilis*; T3, *Pseudomonas fluorescens* I; T4, BP + Pfl; T5, BS + Pfl; T6, BP + BS + Pfl; and Control. The *x* axis represents the treatments and *y* axis represents colony forming units (CFU/g*107). Means followed by the same letter differ non-significantly at $P = .05$ according to DMRT.

Table 4. Effect of biohardening in micro propagated banana against BBTD in pot culture.

Treatments	Virus post-inoculated in biohardened plants						Virus pre-inoculated then biohardened plants	
	PI at 50DAI	PDR at 50DAI	PI at 100DAI	PDR at 100DAI	PI at 150DAI	PDR at 150DAI	PI at 50[a] DAI	PDR at 50DAI
T1	26[b]	67.5	60[e]	25	66[e]	23.25	80[c]	6.97
T2	13[a]	83.7	40[b]	57.5	46[b]	46.51	66[a]	23.25
T3	40[c]	50	53[d]	38.3	60[d]	30.23	80[c]	6.97
T4	13[a]	83.7	40[b]	57.5	46[b]	46.51	80[c]	6.97
T5	26[b]	67.5	46[c]	46.5	53[c]	38.37	73[b]	15.11
T6	13[a]	83.7	26[a]	69.7	33[a]	61.62	80[b]	6.97
Inoculated control	80[d]	–	86[f]	–	86[f]	–	86[d]	–
Healthy	–	–	–	–	–	–	–	–

Note: Percent Infection (PI) = ((number of plants exhibiting BBTV symptoms/ total number of plants) × 100). Percent reduction over control (PDR) = ((Percent infection of control – Percent infection of treatments)/Percent infection of control) × 100) based on Yogeeswarudu & Venkata Krishna (2014). DAI, days after inoculation. Values are the means of three replicates. Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at $P = .5$. T1, *B. pumilus* (BP); T2, *B. subtilis* (BS); T3, *Pseudomonas fluorescens* I(Pfl); T4, BP + Pfl; T5, BS + Pfl; T6, BP + BS + Pfl; Control, water-treated plant.

[a]PI extended up to 150 days of observation.

inoculated healthy control. However, there were no significant differences on all growth parameters among the biohardened treatments and their combinations after pre-inoculation with BBTV.

3.5.2. Post inoculation of BBTV on biohardened plants

In the experiment, where BBTV was post-inoculated three months after biohardening, the days taken to express of BBTD symptoms in these plants were varied from 60.57 to 70.50 days between the treatments (Figure 6). The maximum days taken was 70.5 days in T6 (BP + BS + PfI) followed by T3; however, in control plants, the time taken to express

Figure 5. A view of tissue culture banana plants biohardened with bacterial consortia. (A) Plants challenge inoculated with BBTV, three months after biohardening did not express bunchy top disease symptoms; (B) tissue culture plants biohardened 48 h after BBTV inoculation exhibited typical symptoms of banana.

Table 5. Morphological characters for the viral pre-inoculated then biohardened plants measured three months after planting in pots.

Treatment	Height (cm)	Girth (cm)	No. of leaves (*N*)	Phyllochron	Leaf area (cm^2)
T1	19.20^{bc}	5.40^{c}	8.60^{b}	5.60^{c}	556.48^{cd}
T2	15.80^{d}	6.60^{b}	9.20^{bc}	6.20^{bc}	543.32^{cd}
T3	20.20^{b}	6.20^{bc}	9.40^{bc}	5.80^{c}	664.24^{bc}
T4	17.40^{cd}	5.80^{bc}	9.80^{c}	6.00^{bc}	591.56^{c}
T5	20.20^{b}	5.80^{bc}	9.40^{bc}	6.00^{bc}	685.00^{b}
T6	16.60^{d}	6.20^{bc}	10.00^{d}	6.40^{bc}	595.72^{c}
Virus-inoculated control	18.80^{c}	6.20^{bc}	9.20^{bc}	6.80^{b}	605.60^{bc}
Healthy	28.00^{a}	7.40^{a}	7.60^{a}	7.60^{a}	1569.86^{a}
General mean	19.30	6.17	9.17	6.30	726.65
P value	<.0001	<.0001	0.005	0.001	<.0001
F value	58.328	6.073	3.731	4.702	32.509
Significance	**	**	*	*	**

Note: Values are the means of three replicates. T1, *B. pumilus* (BP); T2, *B. subtilis* (BS); T3, *Pseudomonas fluorescens* I (Pfl); T4, BP + Pfl; T5, BS + Pfl; T6, BP + BS + Pfl; Control, water-treated plants.
*Significant at 5%; **Significant at 1%; NS, non-significant.

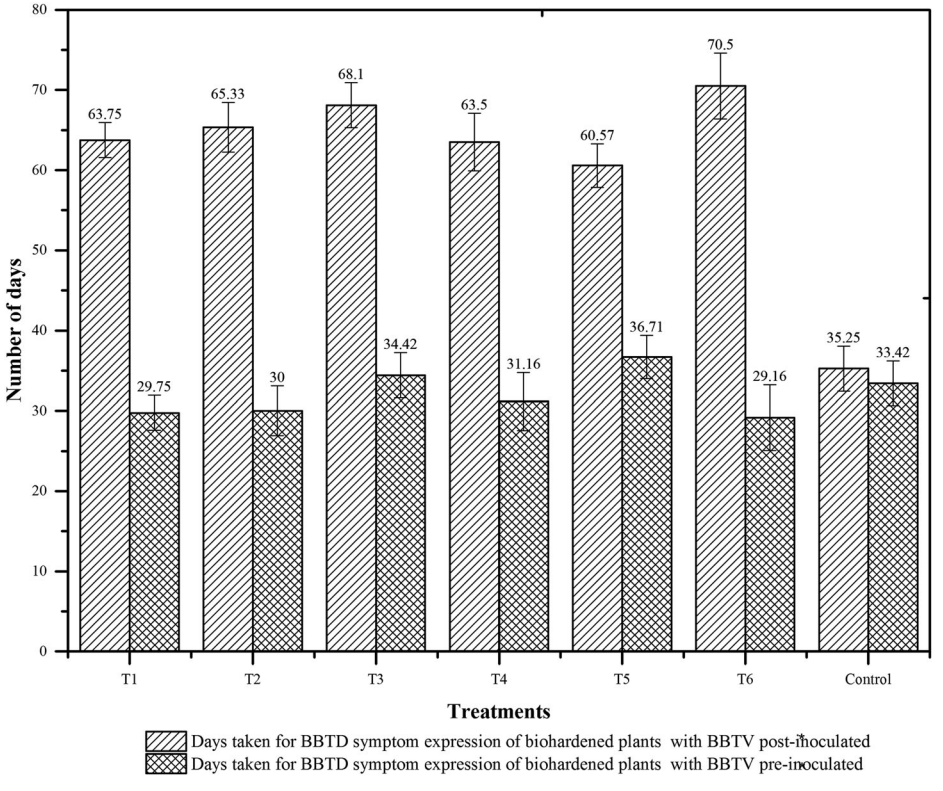

Figure 6. Effect of biohardening tissue culture banana with different combinations of rhizospheric and endophytic bacteria on number of days taken to express BBTD symptoms. *Means are the average of three replicates. T1, *B. pumilus*; T2, *B. subtilis*; T3, *Pseudomonas fluorescens* I; T4, BP + Pfl; T5, BS + Pfl; T6, BP + BS + Pfl and Control. The *x* axis represents treatments and *y* axis represents the days taken for BBTD symptom expression. Bars represent the standard deviation.

the BBTV symptom was 35.25 days. The disease incidence was recorded at 50, 100, and 150 days after inoculation (DAI). Percent incidence (PI) of BBTV at 150 DAI ranged from 33% to 66%, whereas the percent reduction over control ranged from 23.25% to 61.62%. The consortia, T6, showed a maximum of 61.62% PDR over control followed by BS-treated and BP + PfI-treated plants which recorded with 46.5%. The least PDR was recorded in T1 (*B pumilis* treated). In control, 86% of plants expressed symptoms within 35 days after inoculation. In addition to control and delaying the symptom expression of BBTV in biohardened plants, the plant growth parameters have significantly increased than untreated control at150 DAI (data not shown).

4. Discussion

BBTD caused by BBTV is a fatal disease, which is one of the major constraints in banana production worldwide (Almeida & Anhalt, 2008). Eradicating and managing BBTV are very difficult once the virus invades into plantations. Molecular detection tools help in finding out virus-free plants (Selvarajan et al., 2010). Among the popular detection methods, PCR is widely used for the detection of BBTV (Dietzgen, Thomas, Smith, & Maclean, 1999; Manickam, Sabitha, Ganapathy, & Rabindran, 2002). In aspects of controlling, many approaches like controlling of vectors by pesticides (Almeida, Bennett, Anhalt, Tsai, & Grady, 2009) and regular roguing of infected plants from the field have been used (Robson & Jacqueline, 2006). Since pesticides are lethal to ecosystems, in this study we used endophytic bioformulations as an alternative control for BBTD.

Endophytes are beneficial bacteria which live inside the plants. Few studies on a banana and other crops explain that endophytic bacteria can induce the plant growth by secreting useful secondary metabolites, growth hormones in host plants (Beneduzi, Ambrosini, & Luciane, 2012; Mia, Shamsuddin, Wahab, & Marziah, 2010; Van Loon, 2007). Besides, it may provide resistance by inducing systemic resistance in a host against a wide range of pathogens (Salomon, Pinter, Piccoli, & Bottini, 2017). Santoyo, Gabriel, Orozco-Mosqueda, and Bernard Glick (2016) explained that re-inoculating endophytes to roots of the plants triggers a local signal that migrates to the other parts of the plants to activate a systemic enhanced defensive capacity. In this experiment, *Bacillus* spp. and *Pseudomonas* spp. are frequently isolated in the rhizosphere and other parts of banana, which is in conformity with the findings of Thomas (2004) and Thomas and Thyvalappil Soly (2009). The *Bacillus* spp. have shown 100% sequence similarity with *B. pumilus* IHB B 6571 and *B. subtilis* CYBS-4 in BLAST search. Chen et al. (2013) reported that *B. subtilis* CYBS-4 could reduce the wilt disease in tomato. Sharipova et al. (2015) explained that *B. pumilus* ribonuclease possesses antiviral activity against plant viruses such as *Red Clover Mottle Virus*, *Potato Virus X*, and *Alfalfa Mosaic Virus*. Since many authors reported that these two strains were significantly improving plant growth parameters and also having antagonistic properties against various plant pathogens, these two endophytic bacteria were taken for efficacy study (Bogi, Luqman, & Tutung, 2013; Chen et al., 2013; Gutierrez-Manero, Ramos-Solano, Mehouachi, Francisco, & Talon, 2001; Leifert et al., 1995; Xianling, Lu, Gai, Zheng, & Mu, 2008).

In this study, a significant increase in plant defence enzymes such as POX, PAL, PPO, and phenol was noticed in biohardened plants than control plants. Our findings corroborated with the results of Kloepper et al. (2004), Harish et al. (2009a, 2009b), and

Rajamanickam et al. (2018), and they are also in the opinion that the defence enzymes were significantly activated in the biohardened plants, thus strengthening the banana plants to avoid the establishment of BBTV infection. This study was focused to observe the effect of consortia in both pre- and post-viral inoculated plants. A previous study by Harish et al. (2008) explained the effect of their consortia against natural incidence in field level for cv. Virupakshi (ABB). In this study, we have chosen the cv. Grand Naine, which is a prominent cultivar in tissue culture production used all over India, and tested the efficacy of our bacterial isolates in pot culture method. Since the experiments were carried out in pot culture, we have recorded the values only up to 150 DAI of BBTV. Intriguingly, our first experiment (BBTV pre-inoculated and then biohardened) results explained that none of the treaments including consortia did not have any effects against the banana plants and this might be due to initiation of infection process by the virus in those plants. Interestingly, our second experiment results showed that the application of consortia to plants prior to viral inoculation gave 61% reduction in the incidence and also delayed the symptom expressions up to 70.5 days in plants that have expressed the symptoms. These results are in conformity with the findings of Harish et al. (2008). On force inoculation of BBTV by viruliferous aphids, the time taken to induce symptoms varied among all the treatments; also, we got 86% of incidence in control plants. Unknown factors might be affecting or influencing the BBTV establishment in banana (plant self-defence mechanism); this may also be due to the unequal acquisition of virus by aphids from the source plants and dissimilar pattern of transmission of virus to plants (Almeida & Anhalt, 2008; Drew, Moisander, & Smith, 1989; Watanabe, Greenwell, & Bressan, 2013). After 90-100 DAI, BBTV was not detected by PCR in approximately 15% of the newly emerging leaves of non-symptomatic plants of both treatments till 150 days of observation; this may be due to the latency of virus in banana plants (Almeida & Anhalt, 2008; Leclerc, Dore, Gilligan, Lucas, & Filipe, 2014; Watanabe et al., 2013). The latency of BBTV in banana is still an unknown and unresolved phenomenon.

Since the virus invaded prior to biohardening, the phenotype of plants was totally affected, and the physiological parameters could not be correlated. Moreover, the plant growth parameters of biohardened plants were totally decreased compared to untreated healthy control due to the BBTV infection (Hooks et al., 2008). The viral titre was estimated in symptomatic biohardened plants by real-time PCR (data not shown), which revealed that the viral load was higher compared to non-symptomatic but latent plants. This quantitative study of BBTV in biohardened plants before viral inoculation is in agreement with the finding of Harish et al. (2008) that the inoculation of consortia to plants reduces the viral titre. However, our study in BBTV pre-inoculated later biohardened plants did not have similar effect. Based on the results, it could be ascertained that once the virus enters into the plant system, the biohardening process may not have a role in resisting or inducing the resistance mechanism of plants and the expression of symptoms in banana plants. The reason is not known why the same bioagents do not induce resistance in these plants and it is suspected that virus infection might lead to change in the host metabolism leading to hormonal imbalances that may not be irreversible by the bioagents.

So far, the endophytic bacteria–virus relation in plants has not fully understood. We inoculated the bacteria by both root feeding and soil drenching, and the experiments were repeated twice. After three months of biohardening, we estimated the total microbial

load, and the results clearly revealed that total microbial load was higher in biohardened plants compared to non-treated control, which confirms the findings of Nandakumar et al. (2001). They explained that the inoculation of individual bacteria in the banana plant not only induced antagonistic factors but also the other endophytic bacterial colonisation.

Our overall study on bioformulation clearly demonstrated that endophytic *Bacillus* spp. isolated from cv. Grand Naine and re-inoculated to the plants significantly enhanced the growth-related parameters. It also enhanced the induction of defence-related enzymes and pathogen-related proteins in the banana but the single inoculation may not be enough to manage the BBTD. This is the first study carried out to find out the effects of bacterial consortia on BBTV pre- and post-inoculated plants. These results clearly explain the need of pre-treatment of the consortia to delay the symptom expression of BBTD. However, the repeated inoculation at regular intervals with endophyte/rhizosphere bacteria may be required to manage the disease in plants until the bunches are harvested. Based on these preliminary results on the effects of bioformulations of rhizosphere/endophytic bacteria on BBTD, it is assumed that there is a scope of better BBTD management instead of the repeated use of harmful chemical pesticides to control the vector aphids. The plant growth promoting and BBTD control effect cannot be expected if the suckers or corms are from latently infected plants, biohardened with these bacteria identified in this study. In India, nearly 100 million virus-free certified tissue culture plants are being planted annually and if these plants are bioprimed with these consortia then it can protect the plants from BBTV infection and may likely to increase the growth and yield parameters. Therefore, studying the molecular basis of the relationship between these bacterial inoculants and the BBTV might bring a clear understanding of the interactions and would result in effective management of BBTD in future.

Disclosure statement

No potential conflict of interest was reported by the authors.

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