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PLANT TISSUE CULTURE

Phytostimulatory and hardening period-reducing effects of plant-associated bacteria on micropropagated *Musa acuminata* cv. Grand Naine

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Abstract The effects of microorganisms on the health and growth of tissue-cultured plants is not well studied. In the current study, treatment of tissue culture-raised *Musa acuminata* cv. Grand Naine plants with culture supernatants of *Pseudomonas putida*, *Pseudomonas flourescens*, and *Bacillus* sp. had beneficial effects on acclimatization and hardening. At the hardening phase, there was an enhancement of various growth parameters of plants treated with microbial culture supernatants from the first week onwards. Interestingly, after a period of 5 wk, the biohardened plants showed significant improvement in number of roots, length of roots, number of root branches, length of shoot, number of leaves, leaf length, and leaf width. Most remarkably, microbial treatments shortened the hardening period to 5 wk, which otherwise could have taken about 10 wk. The results indicate that selected plant-associated microorganisms, particularly *Bacillus* sp., have the potential to augment acclimatization and to improve the quality of plants during the hardening process. Reduction in the length of the hardening period is very important as it translates into process cost reduction and a resultant reduction in per plant cost. Hence, the outcome of the study has commercial and agricultural applications.

Keywords Micropropagation · Biohardening · *Musa* sp. · Banana · Plant-associated bacteria

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Introduction

Bananas (*Musa* sp.) are well-known as energy-rich food with a good content of mineral salts and vitamins (Chandler 1995). *In vitro* micropropagation is widely used for commercial propagation. Tissue culture-raised banana plants have an average hardening period of 12 wk (Vasane and Kothari 2006, 2008). Susceptibility of the plants to environmental stress, the presence of poorly developed stomata, thin cuticles, hyperhydricity, and a partly heterotrophic nature are the common challenges of the banana hardening process (Hazarika 2003; Chandra *et al.* 2010). Because of a wide array of plant protective and growth-promoting features, plant-associated bacteria are considered to have beneficial effects on micropropagated plants during the hardening period (Nowak and Shulaev 2003; Vestberg *et al.* 2004). Endophytic or rhizospheric bacteria have potential for production of ACC deaminase, nitrogen fixation, and phosphate mineralization (Jimtha *et al.* 2014). Their ability to synthesize phytohormones such as indole-3-acetic acid (IAA) is remarkable, as IAA of microbial origin within the plant or in close vicinity of the roots has a regulatory effect on plant growth and development (Zhao 2010). IAA has crucial roles in root initiation, apical dormancy, tropism, and senescence (Okon 1985; Vande Broek *et al.* 2005). Though our understanding of the influence of microbes on plant growth and physiology is in its infancy, recent studies indicated that the plant microbiome functions as the plant's second genome (Turner *et al.* 2013). Identification of potential microorganisms to accelerate the hardening process and improve growth and health of micropropagated,

economically important plants like banana has potential applications in agriculture to be explored.

Many microorganisms with promising effects on micropropagated plants have been reported (Hallmann *et al.* 1997; Nowak 1998; Vestberg *et al.* 2004). Microorganisms have beneficial effects on rhizogenesis, growth, and reduction of hyperhydricity of plants cultured *in vitro* (Frommel *et al.*1991; Burns and Schwarz 1996; Shetty *et al.* 1996; Carletti *et al.* 1998; Nowak 1998; Barka *et al.* 2000; Mirza *et al.* 2001). Inoculation of tissue-cultured plants with microorganisms could eliminate many of the difficulties associated with hardening process. In a recent study, endophytic *Klebsiella pneumoniae* from shoot tip cultures of banana cultivars 'Tropical' and 'Galil 18' was shown to have a significant impact on pseudostem height, number of leaves, and pseudostem diameter when tested on micropropagated 'Prata Anã' banana plantlets (Fernandes *et al.* 2013). Jimtha *et al.* (2014) isolated *Ralstonia* sp. and *Bacillus* sp. from embryogenic cell suspension cultures of banana and showed them to have plant growth-promoting properties including the production of IAA, siderophore, and ammonia and the solubilization of phosphate. Plant growth-promoting properties like IAA production by bacteria (Arshad and Frankenberger 1991) may enable plants to access more nutrients from soil by increasing the root surface area through increased root growth. Plant growth-promoting rhizobacteria have been suggested to improve proliferation of root hairs in *Zea mays*, *Oryza sativa*, and *Avena sativa* (Beneduzi and Passaglia 2011). Encapsulation of banana shoot tips with rhizosphere bacteria (*Pseudomonas aeruginosa* FP10) was found to increase germination rate compared to controls (Ayyadurai *et al.* 2006). A recent study also demonstrated the growth-promoting ability of endophytic *Bacillus* isolated from banana and its potential for use as a growth-promoting microbial inoculum (Andrade *et al.* 2014). However, studies on microorganisms that can reduce the length of the hardening period of micropropagated plants are very limited, and hence, the present study has much significance. In this study, the potential of plant-associated bacteria to enhance growth and to shorten the hardening process in banana to less than 10 wk was explored.

Materials and Methods

Preparation and maintenance of shoot tip cultures. Banana (*Musa acuminata* AAA cv. 'Grand Naine') shoot tip cultures were initiated from suckers that were collected from a farmer's field in Pattambi, Kerala, India. In the laboratory, suckers were trimmed to 3–4-cm^3 pieces containing the intact shoot meristem. They were surface sterilized with 0.1% (*v*/*v*) mercuric chloride for 8 min followed by several washes with sterile distilled water. Leaf sheaths were removed, and the suckers were trimmed further into 0.5–1.0-cm^3 pieces containing intact meristems. Shoot tips were then inoculated onto Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 13.31 μM 6-benzylaminopurine (Sigma-Aldrich Chemicals Pvt Ltd., Bangalore, India), 30 g L^{-1} sucrose, and 7 g L^{-1} agar. The pH of the medium was adjusted to 5.7 using 1 M NaOH or HCl (all routine chemicals were procured from HiMedia, Mumbai, India). The medium was then autoclaved at 121°C for 20 min and poured into culture tubes (25×150 mm). Cultures were incubated at 27°C under a light intensity of 50–60 μmol m^{-2} $s^{-1}$with a 16-h light (fluorescent tube lights—Philips, Mumbai, India)/8-h dark photoperiod. Shoot multiplication was done by splitting shoot clumps into 2−3 pieces and inoculating the pieces onto fresh medium. Multiple meristem cultures were regularly subcultured at 4-wk intervals. After getting the desired number of plantlets from multiple shoot cultures, *in vitro* rooting of shoots was carried out. For this, uniform-sized shoots with a base diameter of 0.5–0.7 cm and at least three leaves were separated and removed from the shoot cultures. Four shoots were transplanted in each bottle containing rooting media (MS + 0.5 mg L^{-1} indole-3-butyric acid).

Transplantation of plantlets to potting medium. Rooted plants with a minimum of three roots and a height of 4–5 cm were removed from culture bottles. The agar sticking to roots was removed by washing with tap water. Rooted plants were planted individually in polypropylene bags (21 cm×15 cm) filled with a soil and leaf mold mixture in the ratio 2:1. A larger, transparent polypropylene bag was then used to cover the plant to maintain humidity. The bags were transferred to plastic tubs filled with water so that one fourth of the bag was immersed in water. After 2 wk, the covering bags were removed, and plants were kept on the floor of a shade house. Each plant was irrigated with 30 mL of a fertilizer solution containing 4 g L^{-1} FACTOMPHOS (ammonium phosphate and ammonium sulfate; available N/P/K/S 20:20:0:13—The Fertilisers And Chemicals Travancore Limited, Kochi, India) and 1 g L^{-1} 'potash' at 4-d intervals. This method resulted in 100% survival and reduced hardening time to 8 wk, vs. the 10 wk required for conventional primary and secondary hardening (unpublished data, Remakanthan A).

Selection and maintenance of plant-associated bacteria. Among various endophytic and plant growth-promoting rhizobacteria isolated and screened for their plant growth enhancement effect, those with consistent performance were selected for the present study (unpublished data, Radhakrishnan EK). This was confirmed by growth enhancement effect in model plants as per our previous study (Jasim *et al.* 2013). Organisms selected for study include *Pseudomonas flourescens* R68 isolated from rhizosphere of *Syzygium*

jambos, *Pseudomonas putida* R79 isolated from rhizosphere of *Phyllanthus amarus*, and the endophytic *Bacillus* sp. CaB 5 isolated from *Capsicum annuum*. Selected organisms were inoculated into 5 mL Luria Bertani (LB) (Bertani 1951) broth and incubated overnight on a shaker at 37°C at 200 rpm. When the optical density of the suspension reached approximately 0.6 at 540 nm, 100 μL of the suspension was inoculated into 50 mL LB medium containing 0.2% tryptophan in 250-mL conical flasks. The cultures were maintained on the shaker at 37°C and at 200 rpm for 10 d. The cultures were then centrifuged at 1817×*g* for 10 min, and the supernatant was stored under refrigerated condition until used.

Inoculation of bacterial culture supernatant during hardening. For studying the effects of the culture supernatants during the hardening phase, 500 μL aliquots were applied at the base of each plant immediately after placement in the polypropylene bag. Three more applications of supernatant were made at 1-wk intervals. Each treatment group and control group consisted of 10 plants. There were two control groups: one treated with 500 μL LB medium (control without tryptophan) and the other treated with 500 μL LB medium containing 0.2% tryptophan (control with tryptophan). Growth was monitored daily, and data on growth parameters were collected weekly for 5 wk. Subsequently, five plants from each group were uprooted, washed off the soil, and blotted on a blotting paper, and growth parameters (number of roots, length of longest root, number of root branches, fresh weight of plant, fresh weight of roots) were recorded. The experiment was repeated twice, and pooled data were used for statistical analysis.

Statistical analysis. Data were represented as mean ± error of the replicates. The data were analyzed by one-way ANOVA, and means were compared using Duncan's multiple range tests. All the data analysis was carried using "SPSS for Windows13.0" statistical package.

Results and Discussion

Effect of the bacterial culture supernatants on hardening of banana. After 1 wk of hardening, plants treated with bacterial culture supernatants showed significant increase in the height of plants (Table 1). The width of leaves did not vary much, but the number and length of leaves were greater in plants treated with bacterial culture supernatant (Table 1). There were similar responses after 2 wk, and a pronounced increase in plant height and leaf length was observed. Plants treated with culture supernatant of *Bacillus* sp. increased in height significantly when compared to others. After 3rd and 4th wk of hardening, the height of plants and length of leaves were significantly greater in plants treated with bacterial culture supernatant in comparison to the controls.

After 5 wk of hardening, most of the plants treated with microbial culture supernatants were ready for field planting (Fig. 1*F*, *H*, *J*). The observed result was highly significant as the normal hardening period of banana is 10-12 wk. Plants treated with *Bacillus* sp. had the greatest height, but the other two bacterial treatments also resulted in increased height relative to the controls (Table 1). Leaf length and width also were greatest in plants treated with *Bacillus* sp. culture supernatant. Plants treated with *Bacillus* sp. and *P. fluorescens* culture supernatants were comparable for the number of leaves (Table 1). The largest overall plant fresh weight was observed in plants treated with culture supernatant of *Bacillus* sp. (27.01 gm), which was significantly higher than that of the control. The treatments with culture supernatants of *P. putida* and *P. fluorescens* were not significantly different from each other for fresh weight.

Table 1 Effect of the bacterial culture supernatants on growth parameters of banana plants after 5 wk of hardening

Treatments Growth parameters	Control with tryptophan	Control without tryptophan	*Bacillus* sp.	*Pseudomonas fluorescens*	*Pseudomonas putida*
Leaf number	5.30±0.15^{c}	5.10±0.23^{c}	7.45±0.91^{a}	7.10±0.12^{a}	6.40±0.18^{b}
Leaf length (cm)	11.30±0.72^{c}	9.77±0.60^{c}	17.75±0.55^{a}	14.76±0.55^{b}	15.03±0.59^{b}
Leaf width (cm)	5.38±0.34^{c}	4.21±0.24^{d}	8.28±0.31^{a}	6.34±0.25^{b}	6.79±0.31^{b}
Height of the plant (cm)	20.30±1.08^{c}	16.14±0.60^{d}	28.34±0.64^{a}	23.77±0.93^{b}	24.22±1.09^{b}
Number of roots	8.40±0.50^{b}	8.80±0.58^{b}	13.80±0.58^{a}	13.20±0.58^{a}	9.80±0.73^{b}
Length of longest root (cm)	25.20±3.24^{c}	14.20±2.10^{d}	45.00±1.22^{a}	32.30±1.71^{b}	36.10±1.61^{b}
Number of root branches	114.00±10.41^{c}	73.00±13.92^{d}	223.00±4.35^{a}	235.00±13.22^{a}	172.00±9.69^{b}
Fresh weight of plant (gm)	13.28±2.36^{c}	8.68±0.53^{d}	27.01±1.05^{a}	17.44±1.98^{bc}	18.90±0.39^{b}
Fresh weight of roots (gm)	5.86±1.46^{cd}	3.50±0.20^{d}	12.39±1.22^{a}	7.82±1.13^{bc}	9.05±0.24^{b}

Mean values followed by same *letters* are not significantly different within a *row* at $P \geq 0.05$ as determined by Duncan's multiple range test

Figure 1 Hardened tissue-cultured banana plantlets (*Musa acuminata* cv. Grand Naine). (*A*) Experimental setup used for hardening of banana plants. (*B*–*K*) Hardened plants and their roots after 5 wk: (*B*, *C*) control plants treated with LB medium, (*D*, *E*) control plants treated with LB medium containing tryptophan, (*F*, *G*) plants treated with culture supernatant of *Pseudomonas fluorescens*, (*H*, *I*) plants treated with culture supernatant of *Pseudomonas putida*, (*J*, *K*) plants treated with culture supernatant of *Bacillus* sp. *Bars*= 1 cm.

Bacillus sp. and *P. flourescens* bacterial culture supernatant-treated plants had more roots (13.80±0.58 and 13.20±0.58, respectively) compared to controls (8.40± 0.50 and 8.80±0.58) (Table 1, Fig. 1*G*, *K*). All bacterial treatments resulted in significant increases in root length compared to both control treatments. Root length was higher in plants treated with supernatant of *Bacillus* sp. (45.00±1.22 cm) when compared to control (Table 1). Root lengths in plants treated with culture supernatants of *P. flourescens* and *P. putida* were not significantly different to each other. A significant increase in number of root branches was observed in plants treated with bacterial culture supernatants when compared to control (Table 1, Fig. 1*C*, *E*, *G*, *I*, *K*). Out of the three bacterial treatments, plants treated with *Bacillus* sp. and *P. flourescens* culture supernatants showed the maximum number of root branches (about 230). Plants treated with *P. putida* culture supernatant also showed a greater number of root branches (172.00±9.69) than the controls. The least number of branches was observed in plants treated with the control broth lacking tryptophan. Root fresh weights in plants treated with *Bacillus* sp. culture supernatant were increased by four times and two times, respectively, compared to both controls. Root fresh weights of plants treated with *P. putida* and *P. fluorescens* supernatants were not significantly different from each other (Table 1).

In a recent study, PGPR application to micropropagated *Musa* plants resulted in increased root length, shoot length, number of primordia committed to leaf development and number of leaves, fresh weight and dry weight, and protein content (Panigrahi *et al.* 2013). Jie *et al.* (2009) reported the importance of the reintroduction of naturally occurring endophytes into tissue-cultured banana plantlets to improve disease suppression, plant growth, and yield. A study conducted by Jaizme-vega *et al.* (2004) also showed positive results of bacterial application in the developmental stages and foliar mineral contents of *M. acuminata* AAA cv. Grand Naine. Results from the present study showed improvement in the hardening of banana *M. acuminata* cv. Grand Naine, as a result of treatment with culture supernatants of *Bacillus* sp., *P. putida*, and *P. fluorescens*. During the acclimatization phase, the main positive effect of bacterial culture supernatant treatments was increased biomass. This suggested that, during this phase, the chemical biology of the selected bacteria might have improved nutrient uptake of the roots and the overall performance of the plant.

The results of the current study confirm the agricultural potential of endophytic and rhizospheric plant-associated bacteria. The process and procedures involved in micropropagation greatly reduce the natural microflora associated with plants, and application of microorganisms as plant probiotics during the hardening process may enable healthy

and rapid hardening. Since tissue culture is widely used for banana multiplication, a study on the effect of beneficial microorganisms on its hardening is highly significant.

Conclusion

The current study shows phytostimulatory and rapid-hardening effects of culture supernatants from cultures of selected bacterial strains on micropropagated banana. The results showed an increase of growth parameters of treated plants from the first week of hardening onwards. After 5 wk, biohardened plants showed improvement in number of roots, length of roots, number of root branches, length of shoot, number of leaves, leaf length, and leaf width. Interestingly, the hardening process for plants treated with bacterial culture supernatants took only 5 wk to produce plants ready for field planting, which otherwise takes about 10–12 wk. Treatment with the endophytic *Bacillus* sp. was superior to all other treatments during hardening. These bacteria could be used in large scale for industrial hardening of tissue culture-raised banana plants, possibly resulting in significant savings in manpower, time, and valuable hardening space. Unraveling the chemical biology of the observed results could provide deeper insight into plant-microbe interactions.

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