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Isolation and evaluation of 'Endophytes' during plant tissue culture enable identification of potential plant growth promoting bacteria

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Abstract
During plant tissue culture, if any bacterial or fungal colonies are observed, they are regarded as 'contaminants' and immediately, the bottles with contaminants are discarded to control their spread. It should be noted that plants are in symbiotic associations with both epiphytic and endophytic bacteria. During tissue culture, endophytic microbes are observed 2-3 weeks after inoculation, around the explants in media without antibiotics. In the present study, we observed growth of endophytes around the banana explants. Three endophytic bacterial colonies were isolated, purified and evaluated for growth promoting activities. Among the three isolates, Banana Isolate 3 has been found to exhibit IAA production capabilities as well as better root growth promoting abilities. This isolate could, after identification and further characterization, could be used for developing a growth promoting microbial consortium.

Keywords: Banana, explant, microbial endophytes, IAA production, growth promotion

Introduction
Plants are accompanied by a wide range of microorganisms, that include both epiphytes and endophytes. These organisms have either positive, neutral or negative effects on the host plants (Cong, Yu *et al.*, 2021) [1]. Beneficial microbes enable production of growth promoting hormones that are essential for plants, confer resistance to both biotic and abiotic stressors (Glick 2012) [3]. Because of these beneficial effects on plant growth, understanding plant-microbe interactions become essential to achieve / enhance sustainable production and productivity. Plant tissue culture is an area of biotechnology where one of the potential applications include in vitro propagation of commercially important crops. In plant tissue culture, the fundamental principle is to culture explants until the hardening stage in a sterile environment for which strict precautions are taken up during the entire process (Quambusch, Pirttilä *et al.*, 2014) [11]. Though sterile conditions are adopted, it is not completely possible to avoid microbial contaminations in culture which include both pathogenic as well as non-pathogenic / beneficial microbes. Although limited attention has been given to beneficial bacteria in plant tissue culture, recent studies highlight the potentially beneficial role of bacteria on plant growth and development viz., production of essential plant growth hormones like auxins, cytokinins, and gibberellins (Orlikowska, Nowak *et al.*, 2017) [10] (Orlikowska, Nowak 2017) [10] as well as conferring resistance to biotic and abiotic stresses. These dynamic interactions challenge the erroneous notions and underscores the potential benefits of bacteria in tissue culture. Recognizing these positive contributions, the term "vitropaths" has been attributed to non-pathogenic bacteria that have been isolated and proven beneficial for *in vitro* explants (Herman 1987) [5]. Bacteria that were isolated from plant tissue culture reveal a diverse microbial community coexisting with plant cells *in vitro*. This community includes a wide range of bacterial taxa, representing various species and genera (Orlikowska, Nowak 2017) [10]. These bacteria originate from the original plant material used as explants to initiate cultures. Some of these bacteria exhibit intriguing interactions with the plant cells. They may influence the growth rates of the plant tissues or even affect their developmental patterns (Kumar, Jain *et al.*, 2020) [7]. Studying the beneficial interaction can lead to exploring untapped potential of bacterial-plant partnerships (Santoyo, Moreno-Hagelsieb *et al.*, 2016) [12]. In the present study, we report the isolation of three bacterial endophytes associated with

the explants of the banana variety Nendran. Also, we report their utility in promoting root / shoot growth after seed germination in the tomato variety PKM 1.

Materials and Methods

Plant materials: Banana variety, Nendran (AAB) was used in this experiment. Healthy sword suckers were obtained from a farmer's field in Theethipalayam village, Coimbatore district. Two to three months old suckers were collected and transferred to the Plant Tissue Culture Laboratory of the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, where all subsequent experiments were carried out.

Tissue culture media: Modified Murashige and Skoog (Murashige and Skoog 1962) [9] medium that was supplemented with sucrose (30 g/l), agar (0.5%) and 6-benzyl amino purine (BAP) was used for carrying out the experiments. Before autoclaving, the pH of the culture medium was adjusted to 5.8 using a solution of 1N NaOH / 0.5N HCl. Media was then dispensed into jam bottles before being autoclaved at 121 ℃ and 15 psi for 20 minutes.

Plant material preparation: Collected explants were rinsed in tap water to remove the adhering soil particles which was followed by trimming of explants to a length of 4 - 5 cm. Subsequently, the explants were washed with Tween 20 (0.5%) for 20 mins and then washed using RO water thrice for 5-6 min each time. Following this, the explants were subjected to primary and secondary surface sterilization procedures to ensure a healthy *in vitro* establishment. The primary sterilization series was as follows; explants were soaked in 40 mins of 0.5% Bavistin solution (anti-fungal). This is followed by overnight soaking (8-10 h) in ampicillin (0.05%) solution. Then the explants were transferred to an anti-oxidant solution (120 mg/l and 100 mg/l of L-ascorbic acid and citric acid) to counter the effects of oxidation. After every treatment, the explants were trimmed off a layer and washed with autoclaved water thrice. In the secondary sterilization process, the explants were surface sterilized using 70% ethanol and 1.5 % of calcium hypochlorite for 30 seconds and 25 minutes, respectively. After every treatment, the explants were washed with double autoclaved sterile water. Finally, optimally trimmed down explants were inoculated in MS media that supplemented with sucrose (30 g/l), agar (0.5%), and 6-benzyl amino purine (1-10 PPM) and transferred to the culture room.

Examination for growth of endophytes: The inoculated culture media was observed for microbial growth around the explant inoculation site, for up to three weeks. Microbial growth, if observed within 2-3 days after inoculation were considered to be epiphytes (growing after improper sterilization) and were discarded. On the other hand, microbial growth observed after 2 weeks after inoculation were considered as endophytes coming out of plant tissues at the injured ends (during explant preparation). Also, care was taken figure out if theses microbes were affecting the explant growth. Only those microbes that were observed after 2 weeks after inoculation and also, found not affect growth of explant tissues, were used for further experimentation.

Media preparation for culturing endophytes: Nutrient Agar (NA) / Nutrient Broth (NB), a common growth media in microbiology for growing a variety of bacteria was used for isolation and culturing of endophytic bacteria, respectively. For culturing bacteria, NA medium was prepared and autoclaved at 121 ℃ for 20 mins at 15 psi. Then, about 25 ml media was poured into each sterile petri dish and observed for microbial contamination, if any, after three days. Plates free from contamination were used for streaking the endophytic microbial isolates observed in Nendran tissue culture bottles.

Culture isolation: From the Nendran banana tissue culture bottles, an inoculation loop (heat sterilized and cooled) was used for lifting the microbial mat which was then was used to streak on the NA medium plates to enable 'single colony isolation'. After streaking, the plates were incubated in room temperatures (approx. 26-28 °C) to allow growth of bacteria.

Single colony isolation: Incubated plates were observed for morphologically distinct, 'single bacterial colonies. Distinct colonies were lifted and transferred to Nutrient Broth (NB) (10 ml). Then, the bacterial cultures were incubated in a shaker with a setting of 190 rpm and 37 ℃. Then, the cultures were subject to the following assays: IAA production assay and plant growth promotion assay.

IAA production Assay: IAA production assay is used for assessing the ability of the bacteria to produce indole acetic acid which is an indirect estimation of the plant growth promoting ability of the bacteria. The isolates were inoculated in 10 ml Nutrient broth (NB) and incubated in a shaker at 170 rpm at 37 ℃ for 48 hours. The, one (1) ml of each culture was transferred into labeled 1.5 ml centrifuge tubes and centrifuged at 8000 rpm for 10 minutes. Then, to 0.5 ml of supernatant, 1 ml of Salkowiski's reagent was added. The mixture was incubated in the dark for 12 hrs to observe for 'pink colour development' which is an indicator for positive IAA synthesis (Gang, Sharma *et al.,* 2019) [2]. Qualitative data was subjected to statistical analysis.

Growth promotion: Growth promotion assay is used for assessing the ability and identifying the bacteria that promotes root / shoot growth which would be of practical importance in agriculture The isolates were inoculated in 10 ml nutrient broth and incubated in the shaker at 170 rpm at 37 ℃ for 48 hours. One (1) ml of NB was transferred into labeled 1.5ml centrifuge tubes (24 hours after inoculation) to which seeds of the tomato variety PKM1 were added. The seeds were then, kept soaked for 12 h in the bacterial suspension. Seeds soaked in sterile water for 12 hours were used as control. After 12 h, tomato seeds subject to germination and growth promotion test using a 'roll towel' method. The length of the roots and shoots were measured (cms), one week after seed germination.

Statistical analysis

Statistical analyses were carried out using JMP Statistical Discovery (version 10.0.0, SAS Institute Inc., Cary, NC, USA). Both, IAA production and growth promotion assays were carried out in triplicates. ANOVA and Tukey's Honestly Significant Difference (HSD) analyses were performed on the quantitative data obtained on root and shoot lengths.

Results and Discussion

In the present investigation, three (3) endophytes inhabiting the explant tissues of the banana variety Nendran were isolated and subject to IAA production and growth promotion assays. The isolates were named as Banana Isolate 1, Banana Isolate 2 and Banana Isolate 3. The results of IAA production assay and growth promotion assay are given in the Table 1.

IAA production assay

Among the three isolates, only the Banana Isolate 3 was found to have IAA production abilities. On the other hand, Banana Isolate 1 and 2 were not found to have IAA production. This prompted us to confirm whether the banana isolate 3 has good root growth promoting ability and hence, we undertook growth promotion assays.

Table 1: IAA production and Growth Promotion abilities of different banana endophytic isolates

Treatments	PKM 1 Tomato Seed Germination %	IAA Production Assay	Growth Promotion Assay	Shoot Length / Root Length Mean (cm)	Tukey's HSD report			
Control	100	-	Shoot length	4.0	A			
Banana Isolate - 1	100	-		3.6	A			
Banana Isolate - 2	100	-		2.9	A			
Banana Isolate - 3	100	-		3.7	A			
Control	100	-	Root length	4.3		B		
Banana Isolate - 1	100	-		3.4				D
Banana Isolate - 2	100	-		3.5			C	
Banana Isolate - 3	100	+		7.6	A			

Growth promotion assay

All the three isolates were subject to growth promotion assay involving the seeds of the tomato variety PKM1. One week after seed germination [seeds treated with sterile water (control) and banana isolates (treatments)], length of the roots and shoots were measured. It was observed that the average length of the shoots ranged from 2.9-3.6 cms in treatment compared to 4.0 cm in the control. This suggests that none of the isolates performed better in promoting the shoot growth. On the other hand, the root lengths ranged from 3.4-7.6 cm in treated samples compared to 4.3 cm in the control. Among the three banana isolates, Banana Isolate 3 was found to have a mean of 7.6 cm which was 3.3 cm greater than the control samples. Statistical analyses pointed out that the banana isolate 3 is a better performer in case of promoting root growth of the tomato variety PKM1.

For improving overall plant growth, enhancing root architecture is a key factor which is evident in numerous drought related studies. Besides routine plant breeding approaches for enhancing plant growth and yield, application of plant growth-promoting (PGP) bacteria also improves root growth and yield (Ullah, Nisar *et al.*, 2019) [13], which could serve as a sustainable way improving crop production (Hardoim, Van Overbeek *et al.*, 2015) [4]. Plant tissue culturists, who would otherwise discard their contaminated cultures, have an opportunity to explore, isolate and identify potential plant growth-promoting bacteria (Liaqat and Eltem 2016) [8]. These PGPB have a positive impact on micropropagation and biotization (Kanani, Modi *et al.*, 2020) [6] addressing propagation and acclimatization issues, enhancing nutrient availability, environmental sustainability etc., which would be beneficial for the growers. Tissue culture has provided an opportunity to isolate and characterize a number of beneficial endophytes from coconut, anthurium, periwinkle as well as golden pothos (unpublished data). In the present study, out of three endophytic bacteria isolated, one of the isolates have been found to have positive impact on root growth. This provides us with an opportunity to test and use the isolate for plant growth promotion related research and product development.

Conclusion

In conclusion, we recommend that the tissue culture researchers should have an open-minded approach while performing their experiments / commercial tissue culture operations. Identification and evaluation, rather than elimination of contaminating microbes will open doors for identifying potential plant growth promoting microbes of economic significance.

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