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PLANT TISSUE CULTURE

Management of microbial contaminants in the *In Vitro* Gene Bank: a case study of taro [*Colocasia esculenta* (L.) Schott]

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Abstract

Ex situ conservation of vegetatively propagated crops by tissue culture is sometimes hampered by covert endophytic bacteria which become visible after repeated subculture over prolonged periods. In the present study, we identified bacterial contaminants and devised a strategy for their elimination from 20 *in vitro* conserved accessions of taro [*Colocasia esculenta* (L.) Schott] held in the *In Vitro* Gene Bank (IVGB) at ICAR-National Bureau of Plant Genetic Resources (NBPGR). Visually, the cultures were exhibiting white to dark brown exudation in tissue culture growth medium and undergoing slow degeneration as recorded by wilting and necrosis of leaves and shoots. On culturing the macerated pieces of shoots, corms, and leaf petioles from the contaminated taro cultures on bacterial indexing medium, six distinct bacterial colonies were observed. The identity of these bacteria was established through 16S rDNA sequencing as gram-negative strains (2) of *Ralstonia* spp. and gram-positive strains (4) of *Paenibacillus* spp. Thereafter, two strategies were adopted for elimination of contaminants from the cultures: (i) use of antibiotic supplemented media and (ii) hardening of micro-corms in field and re-establishment under *in vitro* conditions. Antibiotic supplemented media was not effective in eliminating contamination as reappearance of bacterial colonies was observed after subculturing in antibiotic-free growth medium. In contrast, re-establishment *in vitro* from hardened infected plants resulted in an average of 6.03% bacteria-free cultures in 14 accessions. The findings indicate that endophytic bacteria associated with the host (taro) plants under tissue culture conditions may efficiently be mitigated through periodic transfer of the tissue culture derived corms to *ex vitro* conditions and re-introduction of the bacteria-free shoot tip explants *in vitro*.

Keywords Bacterial endophytes · Culture revival · *In vitro* conservation · *Paenibacillus* spp. · *Ralstonia* spp.

Introduction

Plant genetic resources are the backbone of crop improvement programs and vital for sustainable agriculture and human survival. Tropical root and tuber crops provide food and nutritional security to a large number of small-hold farmers, especially in developing countries. Taro [*Colocasia esculenta* (L.) Schott], one of the minor root crops, remains a neglected and orphan species with less development of improved varieties as compared to cassava, sweet potato, and yams (Matthews *et al.* 2017). However, its potential to strengthen food security has been recognized. It was grown in tropical Asia for more than 10,000 y (Fullagar *et al.* 2006) and now its cultivation spreads throughout the wet tropics (Matthews *et al.* 2017). Taro is one of the important members of the Araceae family which is consumed as a staple food crop in many countries in the humid tropics and subtropics (Matthews *et al.* 2017). Before the start of the global trade and transport of agricultural

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commodities, taro was the world's most widely cultivated starch crop, extending from India and Southeast Asia to Northeast Asia, the Pacific Islands, Madagascar, Africa, and the Mediterranean region (Matthews 2004). According to FAO databases, taro has the lowest yield among all root crops, with an average of 6.5 tons of fresh corms ha^{-1}. In most countries, taro is grown either as a backyard or home garden crop, or is cultivated by small-holders with very limited inputs. The world production in 2014 was approximately 11 million tons of fresh corms and cormels from 1.5 million ha. However, many countries do not maintain or supply statistics for taro (e.g., India, Bangladesh, Burma, Indonesia, Vietnam, Cuba, and others) although they are significant producers. The highest yields (20 tons ha^{-1}) are obtained in subtropical zones of Egypt and China (FAO 2014). As crops like taro do not feature prominently in organized breeding programs or national seed supply system, the vast majority of farmers rely on their own cultivars or landraces obtained through local selection and traditional exchanges (Lebot *et al*. 2017). In view of this, conserving genetic resources of these crops become important. *In vitro* conservation approaches are the most suitable strategy for *ex situ* conservation of vegetatively propagated crops. Besides being a safe escape from the prevailing threats to biodiversity, including climate change, habitat loss, and cultural change, it offers easy germplasm exchange. In the recent past, there have been a few reports that long-term *in vitro* cultures harbor covert bacteria (Holland and Polacco 1994; Thomas 2004). Their presence hampers international exchange and also becomes a bottleneck for cryobanking of germplasm.

Endophytes are microorganisms that colonize plants internally, often in the intercellular spaces, without imparting any adverse effects (Hallmann *et al*. 1997; Compant *et al*. 2005; Mano and Morisaki 2008). Such bacteria cannot be eliminated by the usual surface-sterilization procedures adopted during plant tissue culture operations (Panicker *et al*. 2007). Thus, isolation of endophytes for identification is generally done after surface sterilization of plant tissue. Presence of endophytes is well documented in micropropagated plantlets of various crops, including watermelon (Thomas 2004, 2007), grapes (Thomas 2006), papaya (Thomas and Kumari 2010), and banana (Thomas *et al*. 2008). The use of antibiotics in controlling endophytes in *in vitro* cultures has been suggested by many researchers (Reed *et al*. 1995; Kulkarni *et al*. 2007; Misra *et al*. 2010). However, these reports lack the information on the probable genetic or epigenetic changes that may occur due to the adverse effects of the antibiotics in the culture medium. Moreover, studies have indicated that some endophytes remain unnoticed with no obvious adverse or pathogenic effect on the host. According to Thomas and Kumari (2010), growth may not be visible in the initial phase of culturing, but outbreaks may be noticed after several years of subculturing, triggered by culture ageing or under the influence of high temperature. These invisible microbial contaminants which co-cultivate with plant cultures are the biggest threats to *in vitro* conservation of germplasm in genebanks throughout the world.

In taro [*Colocasia esculenta* (L.) Schott.], *in vitro* conservation and cryo-conservation are the best-suited approaches for medium-term and long-term storage, respectively. Taro germplasm maintained at the *In Vitro* Gene Bank (IVGB) at ICAR-National Bureau of Plant Genetic Resources (NBPGR) as active collection (90 accessions) comprises many elite types. One lot of accessions (20) was observed as exhibiting loss of regeneration potential with or without visible bacterial contamination (observed as white to cloudy brown exudates) in the culture medium. We could not find any report regarding protocol(s) for elimination of endophytic bacteria from *in vitro* slow-growth cultures of taro (or other species), stored under medium-term duration. Thus, the present work was undertaken to develop protocols for rescue and revival of taro cultures maintained in the IVGB and showing presence of endogenous bacteria.

Materials and methods

Plant material, media and culture conditions Taro cultures initiated from single shoot tips and conserved *in vitro* in the IVGB of ICAR-NBPGR, New Delhi, for 10 to 20 y were used as the starting material. Cultures were maintained in screw cap test tubes (Borosil®, Mumbai, India) containing Murashige and Skoog (MS) medium (Hi Media®, Mumbai, India) (Murashige and Skoog 1962) supplemented with 0.5 mg L^{-1} 6-benzylaminopurine (BAP) (Sigma-Aldrich®, St. Louis, MO), 0.1 mg L^{-1} 1-naphthalene acetic acid (NAA) (Sigma-Aldrich®), 3% (*w/v*) sucrose (Hi-Media®), and 0.8% (*w/v*) bacteriological agar (Hi-Media®), henceforth, referred as slow growth medium (SGM). The cultures maintained on SGM had an extended subculture cycle ranging between three and six mo. For conducting experiments, the shoot-tips were multiplied on the MS medium supplemented with 2 mg L^{-1} BAP, 0.1 mg L^{-1} NAA, 3% (*w/v*) sucrose, and 0.8% (*w/v*) bacteriological agar, herein referred as taro multiplication medium (TMM). For induction of micro-corms *in vitro*, the multiplied shoot-tips were inoculated on medium containing MS salts supplemented with 2 mg L^{-1} BAP, 1 mg L^{-1} NAA, 6% (*w/v*) sucrose, and 0.8% (*w/v*) bacteriological agar, herein referred as high sucrose medium (HSM). The pH of the medium was maintained at 5.8 before being autoclaved at 121°C and 1.06 kg cm^{-2} for 20 min. For experiments with media supplemented with antibiotics, the stock solutions of antibiotics were filter sterilized and added after autoclaving of the media, in laminar air flow (Klenzaids, Mumbai, India). The cultures were maintained at 25 ± 2°C under standard culture room conditions (SCC), subjected to 16-h photoperiod with light

intensity of 40 μE m^{-2} s^{-1}, provided by 40 W cool-white fluorescent tubes (Philips, Mumbai, India).

Bacterial colony isolation and purification-Selection of accessions for isolation of bacteria Out of the total taro germplasm, 20 accessions were selected which had started showing loss of regeneration potential due to tissue necrosis or visually identified with contamination as hazy white, brown, and gray exudates in the culture medium. The accessions were categorized as low (IC87124, IC341446, IC343041, IC334297, IC278228, IC278229, IC317585); medium (IC317643, IC87167, IC87151, IC470444, IC427607, IC427687, IC259024); and highly contaminated (IC86837, IC89512, IC87146, IC89611, IC258963, IC278360) (Supplemental Data). Cultures were scored low (+), medium (++), and highly (+++) contaminated based on visual observations of discoloration of the medium and effect on the growth of the plantlet (wilting of leaf and multiplication rate) when cultured on multiplication medium without any antibiotic (Fig. 3*a*). Plantlets showing exudates in the medium after four wk of subculture were identified. From each accession, at least three tubes were selected randomly for isolation of bacterial colonies.

Isolation of bacteria from varied explants (Bacteria localization) Three different explants, corm, shoot, and leaf petiole (20 each), were excised and surface sterilized using 4% sodium hypochlorite (HiMedia®) for 10 min. After three to four rinses in sterile distilled water, the explants were trimmed and embedded in nutrient agar (NA; HiMedia®) and incubated for bacterial growth at 27 ± 2°C for 48 h in dark. An explant was identified as bacteria positive based on the bacterial growth from any of the indexed parts on the medium. The bacterial colonies were streaked on fresh NA after serial dilution to isolate single colonies with distinct growth pattern, morphology, and color. Gram staining was done using HiMedia® gram staining kit.

Molecular identification of bacteria The single bacterial colonies were picked and multiplied in 5 mL nutrient broth (HiMedia®, Mumbai, India) for 48 h at 28 ± 1°C in an incubator shaker (Labnet International Inc., Edison, NJ) at 150 rpm. Total genomic DNA of bacterial colonies was extracted using CTAB method (Wilson 1987). RNase (HiMedia®) treatment was given to the purified DNA. The purified DNA was quantified by measuring their absorbances at 260/280 nm using Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the final concentration was adjusted to 25 ng μL^{-1} for PCR amplification.

Universal 16S rRNA bacterial primers 8 F (5'-AGT TGA TCC TGG CTC AG-3') and 16S rRNA 1492 R (5'-ACC TTG TTA CGA CTT - 3') were used to amplify this gene using 25 ng of genomic DNA (Sambrook *et al.* 1989). PCR was performed in a 12.5-μL reaction mixture containing 1x-PCR buffer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 1.6 mM $MgCl_2$ (G-Bioscience, St Louis, MO)], 15 mM dNTPs mixture (G-Bioscience), 5 μM each primers (IDT, USA), 1 unit *Taq* DNA polymerase (G-Bioscience), and 25 ng DNA template (G-Bioscience). The temperature cycling program was as follows: initial denaturation at 94°C for 3 min; followed by 37 cycles of 94°C for 45 s, annealing at 42°C (first 5 cycles) and 50°C (for next 32 cycles) for 45 s, and 72°C for 90 s; and a final extension of 72°C for 10 min. The PCR amplifications were performed using a GenePro PCR Thermal Cycler (Bioer, Portsmouth, NH). The amplified PCR products were resolved by electrophoresis on 1.2% agarose (G-Bioscience) for two h in 1x TBE buffer (HiMedia®) at 80 V, stained with ethidium bromide (1 μL) (G-Bioscience) and photographed using the Gel® Documentation System (AlphaImager Corporation, San Jose, CA). PCR product of 1,390 bp amplified by 16S rRNA primers was eluted from the gel using QIAquick gel extraction kit (Qiagen, Germantown, MD) following manufacturer instructions. The rRNA 16S gene from bacterial isolates was sequenced and edited by outsourcing (M/s Invitrogen Sequencing Services, Gurgaon, India). The ribosomal sequence obtained was compared with those deposited in GenBank (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/) using the Nucleotide Basic Local Alignment Search Tool (BLAST) program.

Elimination of contaminants from the cultures Subsequent to identification of bacteria in the cultures, two strategies to rescue the contaminated cultures of taro were tested; one with incorporation with antibiotics in the medium and second comprising field hardening and re-introduction to *in vitro* conditions (Fig. 1).

Addition of antibiotic in the media-Addition of Broad-Spectrum Antibiotics in Media: Rifampicin and Cefotaxime (Sigma- Aldrich®) at different concentrations (50, 100, 150, and 200 ppm) were supplemented in the taro multiplication medium (TMM) to test the effectiveness in controlling the bacterial growth.

Addition of Pathogen-Specific Antibiotics in Media: Pathogen-specific antibiotics (Sigma- Aldrich®), namely streptomycin, oxytetracycline, streptocycline, and chloramphenicol at 50, 100, 150, and 200 ppm, were added in the TMM. Shoot tips (0.5 to 1.0 cm long) with a small corm piece isolated from the contaminated cultures in SGM were cultured on TMM. Multiplied shoot-tips trimmed to about 0.5 to 1 cm were then transferred to the same medium supplemented with antibiotics after surface sterilization using 4% sodium hypochlorite for five to six min and incubated at 25 ± 2°C under a 16-h photoperiod. The cultures of taro growing on antibiotic supplemented media were examined for any visible or invisible

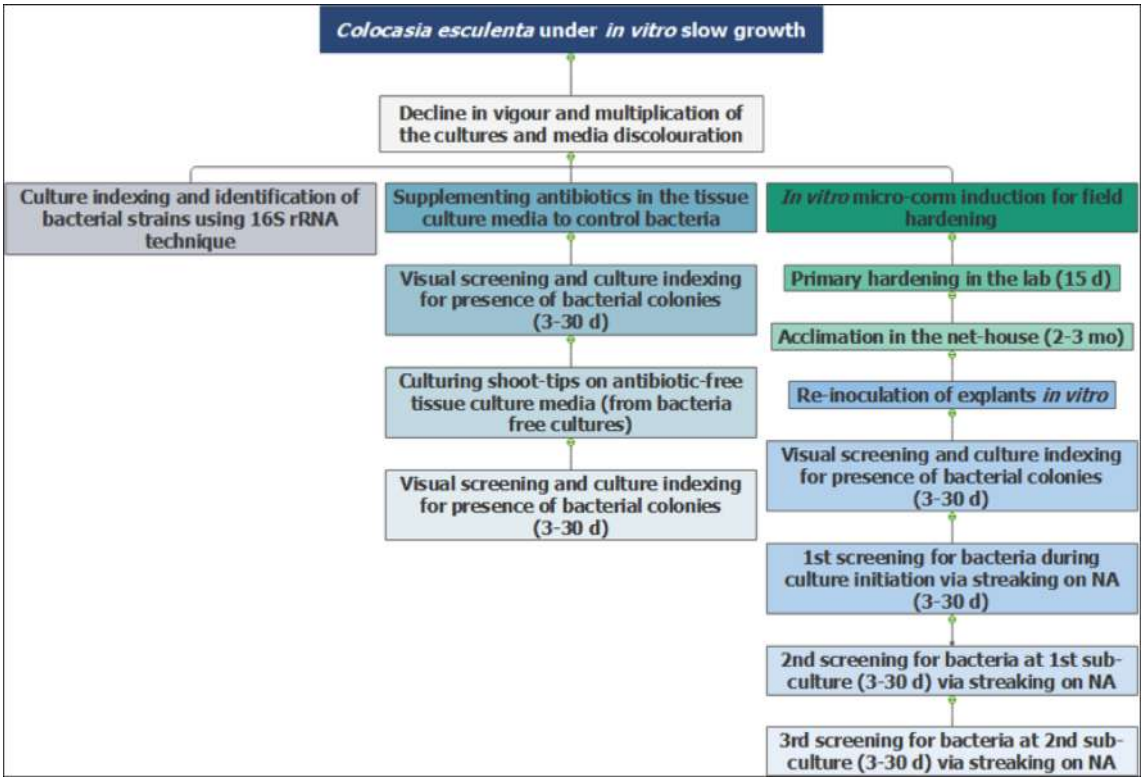

Figure 1. Experimental schema for cleaning infected cultures of *in vitro* conserved accessions of *Colocasia esculenta* (L.) Schott.

infection through visual inspection and indexing every week for one mo, respectively. Two accessions each from low, medium, and high contaminated group (based on visual scoring) were selected for experimentation. Each treatment consisted of three replications with 12 test tubes per replication each containing single shoot tip.

Field establishment and re-introduction *in vitro* An alternative strategy adopted was transfer of the rooted plantlets and *in vitro* micro-corm inducted plantlets to the field, followed by subsequent re-introduction of explants *in vitro*. For rooted plantlets, the plantlets under TMM medium with roots were used for hardening, and for micro-corm induction, the shoot tips isolated from the plantlets maintained in TMM were shifted to a high sucrose medium (HSM). After the induction of micro-corms (within four mo) in all the infected accession, plantlets were hardened in a sterilized mixture of soilrite:soil:sand (2:1:1) and covered with a perforated polybag, for 15 d (primary hardening) followed by transfer to the net house (secondary hardening). Twenty plantlets per accession with preformed micro-corms, as well as without micro-corms, were hardened in 30-cm diameter earthen pots. In each accession, after two to three mo, all the surviving plantlets were re-introduced to the *in vitro* conditions. The explants were disinfected using two step sterilization. First, the corms were washed thoroughly in tap water, and soil debris and outer corm tissues were removed followed by cutting the corm containing shoot tip in to 5-cm^3 piece. The isolated blocks were stirred continuously in solution containing 1% cetrimide with few drops of Tween® 20 (HiMedia Laboratories, Mumbai, India) and 0.1% Bavistin (Carbendazim 50%, Agriplex Pvt Ltd., Bengalurur, India) for 20 min. This was followed by five to six washes with sterile distilled water. Again, the corm pieces are treated with 4% sodium hypochlorite for 15 min in laminar air flow, followed by four to five washes with sterile distilled water. The outer tissues were trimmed and only one to two cm long explant was isolated for culturing. Only those explants which were free from any bacterial contamination (tested via streaking on NA medium) were further utilized for multiplication.

Data recording and analysis Growth parameters including multiplication rate, mortality, leaf senescence, and tissue necrosis due to toxicity of antibiotic concentration were recorded after four to five wk of culture. Shoot multiplication rate in the *in vitro* cultures of taro under slow growth medium without antibiotics, multiplication medium with antibiotic (150 ppm streptocycline) and fresh cultures after field acclimation and re-introduction *in vitro* were calculated as number of new shoots regenerating per explant. The experiments were laid in completely randomized design and data were analyzed using single way ANOVA with *post hoc* test using SPSS. For experiments related to *ex vitro* acclimatization of the micro-corms, 20 plantlets per accession were transferred to the

field and data on survival recorded after two to three mo. The plantlets re-introduced to the *in vitro* cultures were tested for bacterial contamination via streaking the cut end of the explant on NA during subculture. The data of bacterial colonies on NA were recorded after two to three d of incubation at 27 ± 2°C in the dark. At every subculture cycle, the streaking of cut end of the explant on NA was done up to third sub-culture cycle, to test for the presence of bacteria. Field acclimation of micro-corm induced plantlets and re-introduction to *in vitro* conditions was done (Fig. 1) and the data of field survival and percent clean cultures reintroduced *in vitro* were recorded for consecutively three y.

Results

Isolation, identification, and localization of bacteria The surface-sterilized explants after inoculation in the culture medium showed bacterial growth *in vitro* which suggested association of deep-seated endophytes. Bacterial endophytes isolated from different plant parts (pieces of corm, root, and leaf petiole) after dilution plating on NA exhibited six distinct colonies. Out of these, two isolates were found to be gram negative while four isolates were gram positive. The PCR sequencing using bacterial 16S rDNA-based primers yielded amplifications of approximately 1.4 Kb in all the six isolates, which were sequenced using 8F primer, resulting in 1,317-1,390 bp sequences (Table 1).

Partial sequence data of 16S rRNA gene of bacterial isolates are deposited with the NCBI Genbank (see Table 1 for accession numbers). BLAST search analysis of sequences showed 100% similarity of two bacterial isolates with *Ralstonia* spp. (TarInBa 5 and 6) and four bacterial isolates with *Paenibacillus barcinonensis* (TarInBa 1, 2 and 3) and *Paenibacillus* sp. (TarInBa 4). Both strains of *Ralstonia* spp. were found to be mobilized throughout the plant system while *Paenibacillus* spp. was localized only in root and corm tissues (Table 1).

All the 20 infected accessions of taro (Supplemental Data) were categorized based on the intensity of bacterial contamination as low, medium, and highly contaminated cultures. Intensity of infection was observed as the discoloration of the medium as well as the decline of the plantlet vigor and multiplication rate. In some cultures, only white exudates were observed in the medium, while in other accessions, browning of the medium was observed. Mostly, the accessions which showed relatively good shoot multiplication rates were harboring *Paenibacillus* spp. while those accessions detected with the presence of *Ralstonia* spp. showed severe decline in growth and multiplication rate. Cultures showing only hazy white exudates in the medium and found to have *Paenibacillus* spp. either alone or even in the presence of *Ralstonia* spp. were observed to have high vigor and multiplication rates (Table 2). The plantlets which harbor only *Paenibacillus* spp. showed higher multiplication rate (3.25 to 3.54) as compared to the accessions harboring both *Paenibacillus* spp. and *Ralstonia* spp. (1.33 to 2.71).

Table 1. 16S rRNA gene sequence-based identification of Tar series of endophytic bacteria isolated from *Colocasia esculenta* (L.) Schott (taro) shoot-tip cultures

Isolate and NCBI accession number	16S rDNA (bp)	Closest match from NCBI to genus/species level	Suggested identity and accession number of closest match	Description based on growth on NA after 48 h in dark at 27 ± 2°C
TarInBa 1 KT359571	1,390	*Paenibacillus* sp. (B1) (Firmicutes)	*Paenibacillus barcinonensis* 99.28% MG778850.1	Light cream colonies, gram-positive, isolated from root and corm
TarInBa 2 KT359572	1,339	*Paenibacillus* sp. (B2) (Firmicutes)	*Paenibacillus barcinonensis* 99.24% CP054614.1	White colored colonies, gram-positive, isolated from root and corm
TarInBa 3 KT359573	1,360	*Paenibacillus* sp. (B3) (Firmicutes)	*Paenibacillus barcinonensis* 99.26% MG778850.1	Light cream colonies, leaky growth, gram-positive, isolated from root and corm
TarInBa 4 KT359574	1,338	*Paenibacillus* sp. (B4) (Firmicutes)	*Paenibacillus* sp. 99.09% KU891834.1	Yellow colored, round growth colonies, gram-positive, isolated from root and corm
TarInBa 5 KT359575	1,326	*Ralstonia* spp. (B5) (β-proteobacteria)	*Ralstonia* spp. 100% MK459537.1	Light brown colored colonies, gram-negative, isolated from root, corm and leaf petiole
TarInBa 6 KT359576	1,317	*Ralstonia* spp. (B6) (β-proteobacteria)	*Ralstonia* spp. 100% MK459537.1	Light brown colored shining colonies, gram-negative, isolated from root, corm and leaf petiole

Table 2. *Colocasia esculenta* (L.) Schott accessions grouped according to the severity of contamination and bacterial colonies detected (in root and corm) along with shoot multiplication rate after three subcultures in taro multiplication media

Accession ID	Color of the media	Level of contamination*	Number of shoots/explant[1]	Plant height (cm)[2]	Bacteria identified in the cultures
IC341446	Hazy white exudates in the medium	+	3.54 ± 0.14^{a}	5.89 ± 0.03^{a}	*Paenibacillus* spp.
IC343041	Cloudy light yellow colored medium	+	2.71 ± 0.17^{bc}	5.58 ± 0.02^{ab}	*Paenibacillus* spp. and *Ralstonia* spp.
IC278228	Hazy white exudates in the medium	+	3.25 ± 0.20^{ab}	5.22 ± 0.08^{b}	*Paenibacillus* spp.
IC317643	Cloudy light brown medium	++	2.00 ± 0.14^{d}	5.01 ± 0.11^{b}	*Paenibacillus* spp. and *Ralstonia* spp.
IC470444	Cloudy light brown medium	++	2.54 ± 0.29^{cd}	4.15 ± 0.23^{cd}	*Paenibacillus* spp. and *Ralstonia* spp.
IC427687	Cloudy light brown medium	++	2.25 ± 0.12^{cd}	4.38 ± 0.37^{c}	*Paenibacillus* spp. and *Ralstonia* spp.
IC89512	Gray to dark brown colored medium	+++	2.40 ± 0.26^{cd}	3.28 ± 0.17^{cf}	*Paenibacillus* spp. and *Ralstonia* spp.
IC86837	Gray to dark brown colored medium	+++	1.16 ± 0.16^{e}	3.52 ± 0.14^{ef}	*Ralstonia* spp.
IC89611	Gray to dark brown colored medium	+++	1.20 ± 0.14^{e}	3.13 ± 0.23^{f}	*Ralstonia* spp.
IC278360	Gray to dark brown colored medium	+++	1.33 ± 0.08^{e}	3.82 ± 0.09^{de}	*Paenibacillus* spp. and *Ralstonia* spp.

*Low (+), medium (++), and high (+++) contamination degrees are based on visual observations of discoloration of the medium and its effect on the growth of the plantlet (wilting of leaf and multiplication rate) when cultured on multiplication medium without any antibiotic

[1] Data represents mean ± standard error, calculated as average number of new shoots regenerated per explant, 4 wk after third subculture on TMM. Values followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test ($P < 0.05$)

[2] Data represents mean ± standard error, calculated as average length of shoots formed 4 wk after third subculture on TMM. Values followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test ($P < 0.05$)

Effect of antibiotics in cleansing the cultures and growth Treatment with broad spectrum antibiotics in different concentration was found ineffective to control the contamination *in planta*. Thus, after identification of the bacteria, pathogen-specific antibiotics were tested to control the bacterial growth *in vitro*. Among four antibiotics tested at four different concentrations, 150 ppm streptocycline when supplemented in the multiplication medium helped to improve multiplication rate and growth of the plantlets even in the presence of pathogenic bacteria in the system (Fig. 2) as compared to the explant growth in multiplication medium without antibiotics. Initially, the contamination was suppressed, but bacterial colonies re-appeared in the cultures after subsequent subculture of isolated shoot-tips in antibiotic-free medium. Initially up to four wk, 60% of cultures showed no exudates in the multiplication medium supplemented with 150 ppm streptocycline but were found to harbor endophytes upon indexing (Table 3). None of the treatments were able to successfully eliminate bacterial contamination of taro *in vitro*.

Induction of micro-corms, field transfer and re-introduction *in vitro* *In vitro*-rooted plantlets, as well as micro-corm-induced plantlets, were transferred to the pots (in a net house) as an alternative strategy to rescue infected plantlets of taro. For this, the micro-corms were first induced *in vitro* followed by rooting on HSM; the plantlets after four mo of corm induction were shifted to the lab conditions (covering the hardening trays with perforated polybags) for primary hardening, followed by secondary hardening in a net house (Fig. 3). The plantlets transferred to the pots without preformed micro-corm succumbed to death due to dual stress of internal bacterial infection as well as transplant shock (data not presented).

The plantlets with micro-corm survived acclimation phase and survival ranged from 35.3 to 36.8% as per the data of 3 y (Table 4). In the first year, around 400 plantlets (from 20 accessions) with corm were shifted to the net house in August, 2016, out of which only 147 plantlets (of nine accessions) survived in the field, and only 6.12% clean cultures could be established *in vitro*. In the second year (2017), 143 plantlets survived in field out of which only 6 (4.19% of the inoculated explants) cultures were regenerated as clean cultures. In continuation, 141 plants survived in the field during third year (2018) trial and recovery of around 7.8% clean cultures was obtained. If the average of all the three y is viewed, out of 400 plantlets transferred in the field, only 35% plantlets survived in the field, out of which an average of nearly 6% plantlets could be introduced *in vitro* as clean cultures. This indicates that if 100 plantlets will be transferred in the field, the probability of obtaining clean shoot-tip explants *in vitro* is only two to three. Also, it was observed that

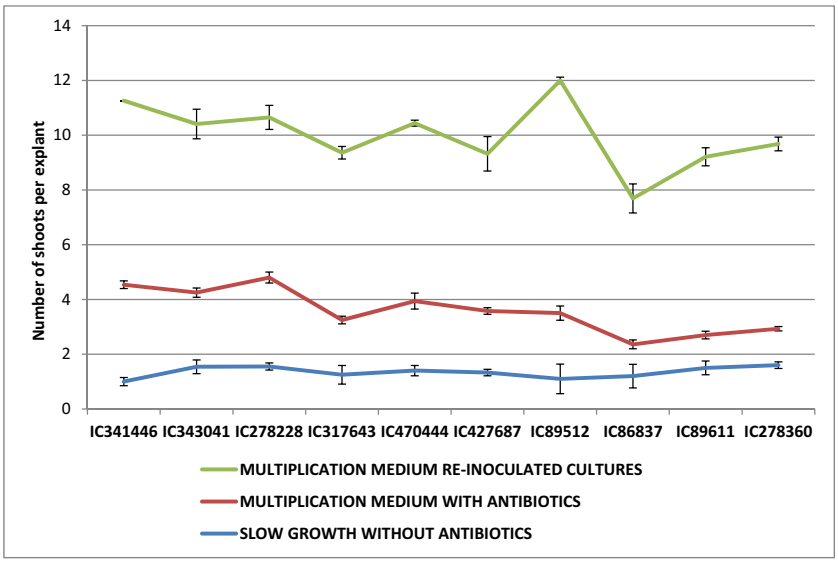

Figure 2. Shoot multiplication rate in the *in vitro* cultures of *Colocasia esculenta* (L.) Schott under slow growth medium without antibiotics, multiplication medium with antibiotic (streptocycline 150 ppm), and fresh cultures after field acclimation and re-introduction *in vitro*.

the accessions which were infected with *Ralstonia* spp. though survived in the field but could not be re-introduced as clean cultures *in vitro* due to visible bacterial growth during culture indexing in the initial inoculation stage.

In our study, we started with 20 accessions for asepsis using the strategy of field introduction and re-inoculation *in vitro*. In the first year, nine bacteria-free accessions could be obtained. In the second year, out of six accessions recovered as bacteria-free, only three were different from the first-year batch of nine accessions. In the third year, out of 11 accessions successfully recovered, only two were new. Thus, in total, 14 accessions (out of 20) could be successfully re-introduced as bacteria-free cultures.

Discussion

This study describes the occurrence of covert bacterial contamination in taro cultures conserved for two decades under slow growth conditions *in vitro* and explores the possibility to exclude the contaminants through various means. Many reports suggest the seriousness of latent or covert bacterial contamination in plant tissue cultures (Leifert *et al.* 1991; Brunner *et al.* 1995; Leifert and Woodward 1998; Leifert and Cassells 2001), which either gain entry as accidental contaminants or as epiphytes or endophytes in the cultures and may survive within the plant system without any symptoms requiring culture indexing for their detection (Viss *et al.* 1991; Thomas 2004).

The *in vitro* cultures of taro showing signs of contamination and media discoloration were found to harbor two types of endophytes in the roots of the plant, *Paenibacillus* spp. which showed growth promoting effects and *Ralstonia* spp. which caused leaf wilting symptoms in plants. In nature, *Ralstonia* spp. is known to cause severe wilting and subsequently death of plantlets by colonizing in vascular bundles in xylem vessels (Schell 2000), whereas, *Paenibacillus* sp. is reported to have a symbiotic association with plants by forming biofilms in the roots (Grady *et al.* 2016). Very recently, Castellano-Hinojosa *et al.* (2018) have reported that *Paenibacillus* sp. also promotes nitrogen fixation, phosphate solubilization, siderophores, and phytohormone indole-3-acetic acid (IAA) production. The endophytic association of *Paenibacillus* has been found in different woody plants like pine, coffee, and poplar (Sakiyama *et al.* 2001; Bent and Chanway 2002). Furthermore, species of this genus are known to produce plant hormones like auxin and cytokinin (Timmusk *et al.* 1999) and hydrolyzing enzymes (Sakiyama *et al.* 2001), as well as peptide antibiotics (Beatty and Jensen 2002), and thus belong to the "plant-growth-promoting Rhizobacteria." Ulrich *et al.* (2008) isolated and identified the *Paenibacillus* strain from the *in vitro* cultures of poplar, larch, and spruce and indicated its potential as plant growth promoting (PGP) endophytic bacteria. Endophytic PGPs have also been documented in many crops which facilitate growth promotion via nutrient solubilization, plant growth hormone production, and nitrogen fixation (Hallman 2001; Bottini *et al.* 2004; Compant *et al.* 2005; Rajamanickam *et al.* 2018). Thus, *Paenibacillus* spp. isolated in this study assumes special significance as beneficial bacteria by improving overall growth of the plant. Apart from PGP activities, the role of *Paenibacillus* as biocontrol agent is also reported. Li *et al.* (2011) indicated that most strains of *Paenibacillus* have the ability to form biofilm *in vitro* and protect tomato seedlings from bacterial wilt caused by *Ralstonia* spp. in the field conditions. They postulated that biocontrol action of *Paenibacillus* strains is through biofilm formation. Recently, Abd Alamer *et al.* (2020) screened different biocontrol agents for antagonistic activity against *Ralstonia solanacearum* and identified one strain of *Paenibacillus polymyxa* (IMA5) as a potential

biocontrol agent to aid the management of bacterial wilt in eggplant with prominent plant growth promoting activity.

In present study, the bacterium *Ralstonia* spp. was found to reduce plant vigor and multiplication, causing severe wilting of the plant and drying of the leaves in addition to the brown discoloration of the growth medium. This belongs to a group of pathogen causing bacterial wilt in many economically important crops including plantain, potato, tobacco, tomato, and groundnut causing severe yield losses (Hayward 1991; Denny 2006). It is known to enter in VBNC state (Viable But Not Culturable) under adverse conditions (Van Elsas *et al*. 2001). Plant pathogenic bacteria generally act through a well-developed enzymatic system to hydrolyze the components of plant cell wall and obtain nutrients and energy, supporting the entry and movement of the microbe in host tissues (Boucher *et al*. 2001). Such bacteria are known to produce high amount of exopolysaccharide (EPS) during in planta multiplication. In case of *Ralstonia* contamination, it has been reported that all the virulent wild-type strains (mucoid colonies) produce EPS (Kelman 1954; Buddenhagen and Kelman 1964; Poussier *et al*. 2003). In plant system, these exopolysaccharides probably cause occlusion of xylem vessels, interfering normal fluid movement of the plant, or by breaking the vessels due to hydrostatic overpressure (Schell 2000).

Symptoms of *Ralstonia* infection include discoloration of the vascular tissue, mainly the xylem, at early stages of infection, and of portions of the pith and cortex, as disease develops, until complete necrosis (Kelman 1953). External symptoms include oozing of slimy viscous substance from the transverse section of the infected stem at the points matching to the vascular bundles (Smith 1896). Blockage of the infected xylem vessel and destruction of the surrounding tissues results in the initial wilting-like symptoms and later death of the plant (Smith

Table 3. Efficacy of antibiotics on growth and multiplication of *Colocasia esculenta* (L.) Schott cultures and suppression of bacteria under controlled conditions

Treatment	Concentration (ppm)	Contamination (%) after 1 mo of culture	Bacterial indexing	Shoot multiplication rate#	Mortality (%)	Remarks
Control	0	100	positive	2.16 ± 0.33^{fg}	39 (38.63)^{d}	White cloudy to brown bacterial buildup in medium within 3 d
Streptomycin	50	100	positive	3.5 ± 0.28^{cd}	20 (26.55)^{f}	White cloudy to brown bacterial buildup in medium within 1 wk
	100	100	positive	3.16 ± 0.60^{cde}	9.66 (18.08)^{g}	White cloudy to brown bacterial buildup in medium within 1 wk
	150	100	positive	3.83 ± 0.16^{bc}	8.66 (17.12)^{g}	White cloudy to brown bacterial buildup in medium within 3 wk
	200	100	negative	0.00^{h}	100 (90.00)^{a}	Injurious to plant growth
Oxytetracycline	50	100	positive	2.16 ± 0.33^{fg}	19 (25.81)^{f}	Cloudy brown medium visible within 1 wk
	100	100	positive	2.45 ± 0.23^{ef}	11 (19.35)^{g}	Cloudy brown medium visible within 1 wk
	150	100	positive	2.83 ± 0.20^{def}	32.33 (34.63)^{de}	White exudates in the medium visible within 3 wk
	200	100	negative	0.00^{h}	100 (90.00)^{a}	Injurious to plant growth
Streptocycline	50	100	positive	4.45 ± 0.33^{b}	0.00 (0.00)^{h}	White cloudy bacterial buildup visible in medium within 1 wk
	100	100	positive	4.03 ± 0.09^{bc}	0.00 (0.00)^{h}	No visible contamination up to 3 wk in 40% cultures
	150	100	positive	5.36 ± 0.29^{a}	0.00 (0.00)^{h}	No visible contamination up to 4 wk in 60% cultures
	200	100	positive	1.5 ± 0.28^{g}	76.66 (61.33)^{c}	Injurious to plant growth
Chloramphenicol	50	100	positive	2.63 ± 0.40^{ef}	31 (33.8)^{de}	Brown growth in the medium visible in medium within 1 wk
	100	100	positive	1.45 ± 0.24^{g}	28.66 (32.35)^{e}	Injurious to plant growth
	150	100	negative	0.00^{h}	93.33 (78.09)^{b}	Injurious to plant growth
	200	100	negative	0.00^{h}	100 (90.00)^{a}	Injurious to plant growth

Tested on 2 accessions belonging to each group; three replications in each treatment, each replication consisted of 12 tubes; Values in parenthesis are arcsine transformed

Data represents mean ± standard error, calculated as average number of new shoots regenerating per explant after 4 wk of culture. Values followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test ($P < 0.05$)

Figure 3. Different stages in retrieval of clean cultures through re-introduction of field acclimatized corms in *Colocasia esculenta* (L.) Schott. (*a*) Cultures showing high, medium, and low contamination. (*b*) *In vitro* micro-corm induction. (*c*) Hardening of corm-induced plantlets. (*d*) Corm harvested from the healthy and disease-free hardened plantlets. (*e*) *In vitro* re-inoculation and selection of bacteria-free cultures. *Bar* = 1 cm (*a*, *b*, *d*, *e*), *Bar* = 5 cm (*c*).

1920; Kelman 1953). *R. solanacearum* is a bacterial plant pathogen which comprises a "species complex" (Fegan and Prior 2005) and possesses several pathogenicity determinants controlled by a density dependent regulatory network (Schell 2000). It enters the plant via the roots, moves through the xylem, and causes lethal disease (Kelman 1953). Under unfavorable conditions, as a survival strategy, many bacteria, particularly gram-negative organisms, enter in to VBNC state (Roszak and Colwell 1987). It was confirmed with different phytopathogenic and symbiotic bacteria including *A. tumefaciens, Rhizobium leguminosarum* (Alexander *et al*. 1999), *Xanthomonas campestris* (Ghezzi and Steck 1999), *Ralstonia solanacearum* (Grey and Steck 2001), and *Erwinia amylovora* (Ordax *et al*. 2006). Such cells have shown the ability to revive, or revert to culturable state, under favorable conditions or with the supply of specific chemicals, metabolites, or host tissue juice (Ordax *et al*. 2006; Ryan *et al*. 2008).

According to Reed and Tanprasert (1995), knowledge of the effect of antibiotics on both bacteria and plant is essential for the recovery of healthy plants. Many workers reported cleansing of infected *in vitro* cultures via isolation, identification of contaminants and pathogen-specific antibiotic

Table 4. Data on field hardening of infected cultures and re-establishment of bacteria-free fresh *Colocasia esculenta* (L.) Schott shoot tip cultures

Year	Number of accessions shifted to field	Number of plantlets shifted to field	Plantlet survival (%)	Number of shoot-tips inoculated *in vitro*	Number of bacteria-free cultures established *in vitro*
2016	20	400	147 (36.75%)	147	9 (6.12%)
2017	20	400	143 (35.75%)	143	6 (4.19%)
2018	20	400	141 (35.25%)	141	11(7.80%)
Average	20	400	143.66 (35.91%)	143.66	8.6 (6.03%)

incorporation to the medium (Tanprasert and Reed 1997; Van den Houwe *et al*. 1998; Thomas and Prakash 2004). Antibiotic treatment is often used against bacterial contaminants (Teng and Nicholson 1997; Misra *et al*. 2010) and for culture cleansing (Kulkarni *et al*. 2007). Misra *et al*. (2010) claimed to cleanse endophytic contaminants from tissue cultured explants (callus) and cultures could be maintained up to two y without any microbial contaminants. In our experiments, we noticed the presence of endophytes in otherwise visibly clean cultures treated with 150 ppm streptocycline and contamination re-appeared after subculturing on antibiotic-free medium. Our results are in agreement with Thomas (2011), who postulated that endophytes in the degenerating cultures of watermelon, which withstood or escape the antibiotic treatment, may remain undetected for about two y on account of their non-culturability and may resume growth in presence of host tissue extract. Thomas (2004) demonstrated drop in rooting potential of cultures of seedless watermelon stocks infected with bacteria after antibiotic treatment. The cultures showed no bacterial growth for subsequent two sub-cultures but found to harbor non-culturable bacteria later. He concluded that cleansing the cultures infected with different bacterial types is complicated. Treatment with antibiotics did not eliminate all the organisms but resulted only in selective suppression of some bacteria, necessitating special approaches to cleanse the stocks.

Thomas *et al*. (2008) reported occurrence of 24 species of endophytic bacteria from *in vitro* cultures of banana surviving in viable but non-culturable state, which turned cultivable after recurrent *in vitro* culturing of stock plants. In another study, Thomas (2011) reported existence of deep endophytic association in 15-y-old cultures of triploid watermelon which were antibiotic tolerant bacteria, initially non-culturable but later multiplied to substantial numbers and turned cultivable. He also mentioned that antibiotics may be a temporary solution to the issue of the endophytic association. The report also emphasized on the activation of these endophytes with extended culture incubation *in vitro*, or at culture revival at higher incubating temperatures than during the conservation phase. Similar observations were made in the present study on taro, where cultures showing contamination and degradation were under routine subculture on slow growth medium since last two decades. The bacterial buildup after every surface sterilization and inoculation *in vitro* affected the overall growth and multiplication of the plantlets and with few plants showing tissue exudates leaching into the growth medium. However, the tissues with no clear symptoms in medium showed foliar discoloration and wilting, suggesting the possible association of the endophyte in the host plant. These explants when inoculated on multiplication medium supplemented with antibiotics showed higher multiplication and growth characteristics as compared to the slow growth medium due to increased cell multiplication rate as compared to the growth of bacterial colonies and effect of antibiotic concentrations. However, when subcultured on antibiotic free medium, bacterial colony reappeared on the growth medium with obvious wilting symptoms in the plants.

In the present study, it was noted that slow growth medium and long subculture cycles resulted in visibility of otherwise non-conspicuous bacterial colonies. Field transfer of such cultures after micro-corm induction for three consecutive years shows that out of approximately 143 explants reintroduced from field, an average of 8.6% clean explants can be reintroduced *in vitro* after field acclimation. An average of 65% plantlets infected with pathogenic bacterium succumbed to death upon field transfer. Re-establisment of cultures *in vitro* from the surviving plants led to 94% elimination during bacterial indexing at the time of culture initiation. Thus, only few explants were recovered free of any type of contaminants; these further utilized to multiply and maintain clean cultures.

This strategy proved promising in re-establishment of the clean explants. The mechanism underlying the survival of *in vitro* multiplied, corm-induced, and hardened plants is probably the exclusion of the pathogenic bacteria in some of the actively growing tissues which ultimately resulted into healthy plants when transferred to field. Also, the plant growth promoting and biocontrol action of *Paenibacillus* spp. may be responsible in suppressing the activity of the *Ralstonia* spp. The probable mechanisms through which the biocontrol agent like *Paenibacillus* works are competition, biofilm formation, induced systemic resistance, and antibiosis.

One of the benefits of field hardening may be the synergistic effects of other microbes, particularly beneficial microbes or naturally occurring endophytic populations present in the soil, reducing the inoculum load in planta. Our study indicated the association of beneficial endophytes in the plant system (*in vitro*), and these endophytes must be isolated and studied for their potential usage in agricultural system to improve plant and soil health as well as their role in disease resistance and improving yields.

Conclusions

Conserving neglected and underutilized species and traditional food crops is one of the best approaches to contribute toward saving local ecosystem and sustainable development. *Ex situ* conservation of vegetatively propagated crops by tissue culture is sometimes threatened by covert endophytic bacteria and become visible after repeated subculture over prolonged periods. In this study, we identified two bacteria in tissue cultured taro germplasm and devised a strategy to eliminate the deep-seated contaminants efficiently, which are not eliminated by extensive surface sterilization as well as incorporation of antibiotics in the media. Re-establishment by field

transfer and selection of field survived plantlets for re-inoculation *in vitro* was found promising and may be utilized in crops having growth characteristics akin to taro. Under aseptic conditions, each explant must be indexed for the probable infection/contamination to eliminate the rest of the infected shoot-tips.

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