

Intense association of non-culturable endophytic bacteria with antibiotic-cleansed in vitro watermelon and their activation in degenerating cultures

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Abstract The study was undertaken with a view to unravel the source of bacterial colony growth observed in a section of micropropagated triploid watermelon cultures that were supposedly cleansed of the associated endophytic bacteria through antibiotic treatment, and thereafter maintained under stringent sterility checks to prevent lateral intrusion of contaminants. Five different bacteria were retrieved from colony growth-displaying watermelon cultures that were previously treated with gentamycin and five isolates from cefazolin-treated stocks with the organisms showing tolerance to the respective antibiotic. These watermelon cultures were in degeneration phase (over 6 months after the previous sub-culturing), while the actively maintained counterpart stocks appeared healthy with no colony growth on different bacteriological media during tissue-screenings. The latter cultures, however, revealed abundant motile, tetrazolium-stained bacterial cells in microscopy, suggesting tissue colonization by non-culturable endophytes. PCR screening on healthy cultures endorsed tissue colonization by different bacterial phylogenetic groups. A few organisms could be activated to cultivation from healthy watermelon stocks through host tissue extract supplementation, which also enhanced the growth of all the organisms. The study indicated that a fraction of antibiotic-tolerant bacteria survived intra-tissue in

non-culturable form during the preceding cleansing activity, multiplied to substantial numbers thereafter, and turned cultivable in degenerating cultures contributed by tissue breakdown products. This study brings out the existence of a deep endophyte association in tissue cultures which is not easily dissociable. It also signifies the utility of in vitro system for investigations into plant–endophyte association and to bring normally non-culturable novel organisms to cultivation facilitating their future exploitation.

Keywords Bacterial endophytes · *Citrullus lanatus* · Endophytic microorganisms · Microbial contamination · Plant tissue culture · Tetrazolium staining · VBNC · Viable but non-culturable bacteria · Videography

Abbreviations

HTE	Host tissue extract
NA	Nutrient agar
TTC	2,3,5-Triphenyl tetrazolium chloride
TSA	Trypticasein soy agar
VBNC	Viable but non-culturable
WMM	Watermelon multiplication medium

Introduction

Contrary to the earlier assumption of asepsis with plant tissue cultures, it is now emerging that in vitro cultures frequently harbor bacteria in a non-obvious or covert form (Holland and Polacco 1994; Herman 2004; Thomas 2004a, b). This association is detected through culture-indexing, i.e., testing the medium and tissue from visibly clean cultures on different bacteriological media (Leifert and Woodward 1998; Thomas

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2004a, b). Endophytic bacteria which colonize plants internally and not eliminated by surface-sterilization constitute a major source of entry (Pirttilä et al. 2000, 2005; Panicker et al. 2007). Recent studies have brought out the widespread association non-culturable endophytes with tissue cultures, which are detected through molecular and microscopic techniques (Thomas et al. 2007, 2008; Podolich et al. 2009; Thomas and Soly 2009).

Following the detection of covert association of bacteria with the long-term micropropagated triploid watermelon culture (Thomas 2004a), our main interest was to cleanse this culture. This was supposedly accomplished through a multi-step approach involving NaOCl treatment of shoot-tip segments and submerged incubation in antibiotic supplied medium (gentamycin or cefazolin) followed by culture-indexing at each sub-culturing employing two bacteriological media. The identified cultures remained consistently index-negative during the next 2 years with the restoration of growth in vitro and normal fertile plants in the field facilitating their labeling as bacteria-cleansed stock (Thomas et al. 2006).

Nearly 3 years after the original antibiotic treatment, some of the sub-cultures from ‘bacteria-cleansed stocks’, which were not into the active multiplication displayed bacterial colony growth emanating from the base of shoots after 6 months of the previous sub-culturing. The origin of colony growths from the base of senescent cultures and the stringent sterility checks adopted during the course of studies suggested that the organisms were not external contaminants. Around this time, information about the widespread association of non-culturable endophytic bacteria with the cultures of papaya and banana emerged (Thomas et al. 2007, 2008), but these cultures were not given an antibiotic treatment unlike the case with watermelon. The present study was taken up with the aim of elucidating the source of bacteria in the expectedly bacteria-cleansed watermelon cultures and to explore further into plant–microbe interaction in supposedly sterile in vitro system.

Materials and methods

Plant material, culture-indexing procedure and sterile practices

The present studies were undertaken during the 11- to 15-year period of the triploid watermelon (*Citrullus lanatus* (Thunb.) Matsum and Nakai) culture originated in vitro during 1995. Description of this stock and other details can be found elsewhere (Thomas 2004a). Watermelon multiplication medium (WMM) in culture bottles (110 × 65 mm) was used for tissue-culturing work with four

shoot-tip or nodal microcuttings (approx. 1 cm) per vessel. Watermelon cultures were transferred to fresh WMM monthly employing 20 culture vessels, for which 6–8 stocks provided the necessary number of microcuttings. Indexing was carried out on such stocks at sub-culturing, which refers to the testing of visibly clean tissue cultures for any covertly associated/endophytic bacteria by transferring traces of culture medium and split tissue segments onto at least two bacteriological media namely nutrient agar (NA) and trypticasein soy agar (TSA), and observing for any colony growth at 37°C (NA) or 30°C (TSA) for the first 2–3 days and thereafter at 25°C for 1–4 weeks (Thomas 2004b; Thomas et al. 2006). Other bacteriological media used during this study included Luria–Bertani agar (LBA) at 37°C and brain heart infusion agar (BHIA), McConkey agar (MA) and 0.1× NA and 0.1× TSA at 30°C. The term culture or stock in this report refers to watermelon unless specified as bacterial culture/cell; the latter are also referred to as motile particles.

During and after the culture cleansing activity described earlier (Thomas et al. 2006), all possible care to prevent further microbial entry was taken keeping in mind the various known routes of microbial intrusion (Thomas et al. 2007, 2008). Additionally, the culture handling tools were autoclaved prior to the use on any day, and in between individual cultures, they were sterilized in a glass-bead sterilizer at 260°C for ≥5 min. The tools prior to tissue-handling were indexed on NA and TSA to ensure effective sterilization. To take care of high heat-tolerant spore formers, double autoclaving of culture vessels for 20 min each, one prior to the medium preparation and the second with the medium, was performed. Post tissue-inoculation, the culture bottles were sealed in polypropylene (PP) bags (7 × 5"), which ensured an aseptic environment outside the culture vessel as well (unpublished results).

All the culture transfer operations were undertaken in an ultra low penetration air (ULPA) filter-fitted laminar with 99.999% sterility assurance (Esco Biotechnology Equipment Division, Singapore). Bacteriological media were prepared in single-use Petri-dishes and were incubated at 37°C for one night followed by 2–3 days at 25–30°C prior to their use in indexing or bacterial isolation to ensure sterility. Post-indexing, the plates were sealed individually in sterile PP bags and observed for bacterial colony growth.

Isolation of bacteria and molecular-based identification

Bacterial growth-displaying old-stocks could be segregated to two categories based on the color of fluidy colony, namely yellow or cream. These were traced to have originated from the previously gentamycin (GM; 100 mg l⁻¹) and cefazolin (CZ; 1,000 mg l⁻¹) treated sets, respectively. Colony growths from two bottles of GM series were pooled

and progressed through serial dilution and plating on TSA and NA. The same was done with CZ series too. After 2–7 days of plating and incubation at 30/37°C, distinct colony morphotypes were identified and carried forward through two rounds of streaking and single colony selection. Five bacterial isolates, each derived from the GM stocks and CZ stocks, were taken up for identification based on partial 16S rRNA gene sequence homology analysis as described (Thomas and Soly 2009). The identity was fixed based on nucleotide blast analysis at the National Centre for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Seqmatch search of type strains at the Ribosomal Database Project (<http://rdp.cme.msu.edu/>) as on 30 August 2011.

Testing the retrieved organisms for antibiotic resistance

The isolates GM01 to GM05 retrieved from gentamycin treated watermelon stocks and CZ01–CZ05 from cefazolin treated sets were challenged against these two antibiotics to assess if they survived in the cultures on account of the antibiotic resistance. Aliquots (5 µl) of single antibiotics at 25, 50, 100, 250, 500, 1,000 or 10,000 mg ml⁻¹ were applied 2 h after plating the respective bacterium on NA (100 µl of 0.1 OD) and observed for any clear zone at 30°C.

Indexing of healthy cultures and microscopic observations

The apparently clean cultures that were in active micro-propagation (20 bottles) were subjected to medium and tissue-indexing using NA and TSA as described earlier (Thomas et al. 2006). Tissue from these stocks were surface-sterilized using NaOCl (2% available chlorine) or 0.05% HgCl₂ for 5 min and the effective removal of all the external organisms was confirmed with tissue imprinting as well as plating the wash solutions on NA and TSA. Tissue was squeezed aseptically onto autoclaved microscope slides and the crush was examined after covering with an alcohol-dipped, autoclaved cover glass under bright field or phase contrast (100× oil objective) using a Leica DM2000 microscope as per Thomas et al. (2007). The images and video clippings were captured using Leica DFC-280 digital live camera with a 6.3× magnifier and Leica Application Suite software (Leica Microsystems Ltd, Heerburg, Switzerland). To ascertain the internal tissue colonization, thin free-hand sections were prepared on a microscope slide from surface sterilized tissue using a razor blade and the sections were mounted in sterile distilled water and examined as above. To confirm that the actively motile particles seen in tissue sections were indeed bacterial cells, vital staining was practiced using 2,3,5-triphenyl

tetrazolium chloride (TTC) as per Bacon et al. (2002). For this tissue was soaked in TTC (1.5 g l⁻¹) malic acid (625 g l⁻¹) solution for 1–7 days and thin free-hand sections were prepared as above under aseptic conditions. The sections were mounted in sterile water and examined under bright field for red stained bacterial cells.

Molecular screening of watermelon cultures with universal and group-specific bacterial primers

To ascertain the association of bacteria with watermelon tissue as normally non-culturable endophytes, PCR amplification using the DNA template isolated from watermelon cultures was attempted employing the primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP1/rP2 (GGYTACCTTGTTACGACTT) targeting most eubacteria (1,502 bp amplicon; Weisburg et al. 1991), and 9bfm (GAGTTTGATYHTGGCTCAG) and 1512uR (ACGGH TACCTTGTTACGACTT) for eubacteria and archaea (1,503 bp; Mühling et al. 2008).

Further, primers specific to different bacterial phylogenetic groups were employed in PCR to ascertain the association of different classes of bacteria as endophytes. These included Alf28f (ARCGAACGCTGGCGGCA) and Alf684r (ACGAATTTYACCTCTACA) for α-proteobacteria (674 bp), Beta359f (GGGGAATTTTGACAATGG) and Beta682r (ACGCATTTACTGCTACACG) for β-proteobacteria (342 bp), Gamma395f (CMATGCCG CGTGTGTGAA) and Gamma871r (ACTCCCCAGGCG GTCDACTTA) for γ-proteobacteria (496 bp) (Ashelford et al. 2002; Mühling et al. 2008), 243f (GGATGAGC CCGCCGCCTA) and 1492r (TACGGGTACCTTGTTA CACTT) for Actinobacteria (1,287 bp; Heuer et al. 1997; Conn and Franco 2004); Firm350f (GGCAGCAGTRGGG AATCTTC) and Firm814r (ACACYTAGYACTCATCG TTT) for Firmicutes (483 bp; Mühling et al. 2008), Bacil47f (GCCTAATACATGCAAGTCGAGCG) and Bacil365r (ACTGCTGCCTCCCGTAGGAGT) for *Bacillus* group (318 bp; Goto et al. 2000) and CYA361f (GGA ATTTCCGCAATGGG) and CYA785r (GACTACWG GGGTATCTAATCC) for Cyanobacteria/chloroplasts (444 bp; Mühling et al. 2008).

For DNA extraction, shoot tissue from index-negative cultures were surface-sterilized and homogenized aseptically in Tris–EDTA, the debris was allowed to settle down and the supernatant, which showed abundant motile bacterial cells, was processed as per CTAB protocol (Ausubel et al. 2005). PCR was set up with different primer combinations in 20 µl reactions employing the basic program as per Mühling et al. (2008) for the primer pair 9bfm/1512uR consisting of an initial denaturation step of 4 min at 96°C, 30 cycles of 96°C for 60 s, 52°C for 90 s and 74°C for 60 s followed by a final extension step at 74°C for

10 min. As a positive check, DNA from different organisms representing α , β and γ proteobacteria (*Rhizobium*, *Xenophilus* and *Sphingomonas*, respectively), actinobacteria (*Microbacterium*, *Cellulosimicrobium*) and firmicutes (*B. pumilus*) were used as control template. Wherever the amplification was not attained, PCR was repeated employing the thermal cycling conditions specified for the primer pair, or using the first round PCR product from 9bfm, and 1512R as the template.

Attempts to bring non-culturable organisms to cultivation

Cultures that were index-negative on NA and TSA were indexed on other bacteriological media (LBA, BHIA, MA, $0.1\times$ NA and $0.1\times$ TSA), and those proving index-negative on various media were used in this experiment. At the onset of the experiment, tissue from four such cultures were homogenized in a mortar (100 mg ml⁻¹ sterile water or nutrient broth) and 100 μ l sample from each was plated on NA and TSA to check for the presence of any culturable organisms. Tissue homogenate and choppings from such cultures were used in the subsequent experiments as described under “Results”. The derived colony growths from two treatments (tissue homogenate prepared in WMM and mixed with $0.1\times$ NB or $0.1\times$ TSB) were further progressed through single colony selection and identification as above.

Effect of host tissue extract on bacterial growth

The 14 isolates retrieved in this study including ten from visible colony growth-displaying senescent cultures and 4 that were activated to cultivation from the index-negative cultures were provided with host tissue extract (HTE) to see if the bacterial growth in aging stocks was related to the release of tissue breakdown products with culture degeneration. HTE from aged in vitro cultures was prepared in sterile distilled water (100 mg ml⁻¹) followed by filter-sterilization (Thomas and Soly 2009). Sterility of the filtrate was ascertained by spotting on NA and TSA. WMM in square disposable-dishes (12 \times 12 cm; Hi Media, Mumbai) was applied with the HTE at 10 μ l cm⁻² (1.44 ml plate⁻¹) and allowed to dry in a laminar. A plate applied with sterile water served as control. The media were inoculated with 5 μ l of dilute bacterial suspension (OD 600 nm of 0.1) prepared in sterile distilled water from 24 to 48 h NA plate cultures. A third plate with NA was employed to ensure the presence of sufficient bacterial inoculum. The plates (three replications per treatment) were incubated at 30°C for two nights and monitored for colony growth.

A quantitative estimate of the extent of growth promotion by tissue constituents was obtained through HTE assay as per Thomas and Soly (2009). For this $0.1\times$ NB in 2 ml microfuge tubes was supplemented with HTE at 100 μ l ml⁻¹ and inoculated with 50 μ l of bacterial suspension of 1.0 OD (1 ml final volume; five replications per isolate) and the OD was assessed on the third day after incubation at 30°C (220 rpm) employing blanks stored at -20°C from ‘time zero’.

Statistical procedures

The experiments were set up keeping in mind the basic principles of completely randomized design with adequate replications, or were repeated for consistency as necessitated or feasible. Quantitative assays like assessing the effect of HTE on bacterial growth were set up in CRD with five replications and the data were subjected to single factor ANOVA employing the data analysis tool of MS Excel 2003. Mean \pm SD values are presented.

Nucleotide sequences

Partial 16S rRNA gene sequences of the 14 isolates are deposited with NCBI Genbank with the accession numbers HM146137–50.

Results

Isolation and identification of bacteria from senescent cultures

Following the cleansing activity, the watermelon cultures showed restoration of normal growth on WMM (Fig. 1). A section of watermelon cultures that were poorly responsive and in degeneration phase displayed bacterial colony growth almost 6–8 months after the previous sub-culturing (Fig. 1-inset). The cultures that were taken through regular monthly sub-culturing, on the other hand, remained healthy and visibly clear of microbial contaminants.

Five different colony morphotypes were retrieved from the yellow colony growth in the previously gentamycin (100 mg l⁻¹) treated set after dilution-plating and they belonged to *Cellulosimicrobium*, *Microbacterium*, *Sphingobacterium*, *Brevundimonas* and *Gordonia* spp. (Table 1). Similarly, the cream colony growth from cefazolin (1,000 mg l⁻¹)-treated set yielded one culture each of *Bacillus pumilus*, *Cellulosimicrobium* sp., *Rhizobium radiobacter* (previously *Agrobacterium tumefaciens*; Young et al. 2001), *B. safensis* and *Xenophilus* sp.



Fig. 1 Healthy and senescent triploid watermelon cultures. A healthy culture subsequent to the original cleansing activity showing normal growth on WMM 1 month after sub-culturing, and (*inset*) a non-responsive senescent culture showing bacterial colony growth emanating from the base after 6 months

(Table 1). Thus, a good number of isolated organisms appeared to be uncommon or novel organisms.

Antibiotic sensitivity assay

The GM-series isolates were unaffected by 100 mg l^{-1} gentamycin, the level employed during cleansing, but were sensitive to higher levels (Table 2). Similarly, the CZ-series isolates from cefazolin treated stocks, in general, were insensitive to this antibiotic except for *B. pumilus*, a spore former, and the Gram-negative *Xenophilus* sp. Most of GM-isolates were insensitive to cefazolin and the CZ-isolates to gentamycin except at high levels (data not presented).

Indexing of healthy cultures and microscopic observations

Indexing of counter-part healthy watermelon cultures that were maintained with regular sub-culturing did not show any colony growth on the seven different bacteriological media employed. On the other hand, tissue squeeze from these cultures revealed abundant motile particles including cocci and oblong cells/fine rods under bright field

microscopy (Fig. 2a; Online Resource 1). Pure cultures of bacteria retrieved from degenerating stocks showed cell motility quite similar to that observed with the tissue squeeze or in tissue segments, particularly in the case of coccoids and fine rods characterized by active ‘wriggling’ movement in the thin film of water (Online Resource 2). Green pigmented chloroplasts and bright mitochondria became more obvious under phase contrast amidst abundant motile phase-dark bacterial cells (Fig. 2b; Online Resource 3). Occasionally phase-bright spores (approx. $1\text{--}2 \mu$) were seen distinct from mitochondria ($3\text{--}5 \mu$). Pooled pure cultures of different organisms isolated from watermelon also showed similar features including cell size and wriggling movement (Online Resource 4).

Free-hand sections from surface-sterilized healthy green tissue of index-negative cultures also showed such motile bacteria amidst chloroplasts (Online Resource 5) whereas tissue from index-positive cultures had them quite in abundance (Online Resource 6). The release of bacterial cells from the tissue was facilitated by the application of a bit of pressure on the cover glass and the active motility became more obvious a few minutes after the incident illumination. Shoot tissue incubated in TTC solution for one night displayed light pink-stained bacterial cells including motile and non-motile ones, and cocci and fine rods, endorsing that the moving particles were indeed live bacteria (Fig. 2c, d; Online Resource 7). A fraction of cells in a field appeared unstained which proved to be the cells not in focal plane. Extended tissue incubation in TTC solution for 4–7 days revealed abundant pink-stained bacterial cells of varying size and shapes, including motile and non-motile ones, trapped inside the tissue and seen in different planes of tissue sections (Fig. 2e; Online Resource 8).

Fewer bacterial cells were detected in the tissue-incubating TTC solution compared with intra-tissue. The TTC solution used for incubating index-negative healthy cultures did not show any colony growth on NA or TSA suggesting that live bacterial cells observed still deemed to be termed as normally non-culturable. More bacterial cells took up staining with the extended incubation in TTC, or possibly some bacterial multiplication took place during this period.

While several of the organisms isolated in this study were cocci shaped (Table 1), fine rods also showed some amount of cocci which proved to be the end view of moving cells (Fig. 2f, g). Pure cultures of organisms incubated in TTC solution showed cell staining within 24 h in some isolates (GM03, CZ04) while others showed low or partial staining (GM01, GM02, GM04, CZ01, CZ02, CZ03) or no staining (GM05, CZ05) (Fig. 2h, i; Online resource 9, 10). Incubation in TTC solution for 2–4 days resulted in pink staining of most bacterial cells while some

Table 1 Identification of endophytic bacteria retrieved from senescent watermelon cultures that were previously treated with antibiotic gentamycin (GM) or ceftazolin (CZ)

No.	Isolate ID	16S seq (bp) and NCBI Acc. No.	Closest NCBI species identity and homology	RDP best match to type- strain and homology	Suggested identification and phylogenetic group	Cell type under phase and Gram reaction
1.	WM-GM 01	753 (HM146137)	<i>Cellulosimicrobium cellulans</i> (GQ503328; 99%)	<i>Cellulosimicrobium funkei</i> (T); W6122 (AY501364; 0.977)	<i>Cellulosimicrobium</i> sp.; Actinobacteria	Motile small rods to cocci; G +ve
2.	WM-GM 02	559 (HM146138)	<i>Microbacterium paraoxydans</i> (HQ591426; 100%)	<i>Microbacterium paraoxydans</i> (T); CF36 (AJ491806; 0.978)	<i>Microbacterium paraoxydans</i> ; Actinobacteria	Motile fine rods; G +ve
3.	WM-GM 03	613 (HM146139)	<i>Sphingobacterium</i> sp. (EU927288; 97%)	<i>Sphingobacterium multivorum</i> (T); IAM14316 (AB100738; 0.834)	<i>Sphingobacterium</i> sp.; Bacteroidetes	Medium rods; G -ve
4.	WM-GM 04	655 (HM146140)	<i>Brevundimonas intermedia</i> (FJ609705; 99%)	<i>Brevundimonas intermedia</i> (T) ATCC 15262 (AJ227786; 0.894)	<i>Brevundimonas</i> sp.; α proteobacteria	Medium rods; G -ve
5.	WM-GM 05	577 (HM146141)	<i>Gordonia terrae</i> . (HM151876; 99%)	<i>Gordonia terrae</i> (T); (X79286; 0.968)	<i>Gordonia</i> sp.; Actinobacteria	Small rods often in pairs; G +ve
6.	WM-CZ01	606 (HM146142)	<i>Bacillus pumilus</i> (JN210575; 100%)	<i>Bacillus pumilus</i> (T); ATCC 7061; AY876289; 0.990	<i>Bacillus pumilus</i> ; Firmicutes	Floating rods and spores; G +ve
7.	WM-CZ02	759 (HM146143)	<i>Cellulosimicrobium funkei</i> (EU977646; 99%)	<i>Cellulosimicrobium funkei</i> (T) W6122 (AY501364; 0.955)	<i>Cellulosimicrobium</i> sp.; Actinobacteria	Small rods to cocci; G +ve
8.	WM-CZ03	509 (HM146144)	<i>Agrobacterium tumefaciens</i> (FR828333; 100%)	<i>Rhizobium radiobacter</i> (T) IAM 12048 (AB247615; 0.895)	<i>Rhizobium radiobacter</i> ; α proteobacteria	Actively moving medium long rods; G -ve
9.	WM-CZ04	727 (HM146145)	<i>Bacillus safensis</i> (JF411317; 100%)	<i>Bacillus safensis</i> (T); FO-036b (AF234854; 0.996)	<i>Bacillus pumilus</i> ; Firmicutes	Floating rods and spores; G +ve
10.	WM-CZ05	662 (HM146146)	<i>Xenophilus aerolatus</i> (JN585329; 99%)	<i>Xylophilus ampelinus</i> (T) DSM 7250 (AJ420330; 0.841)	<i>Xenophilus</i> sp.; β proteobacteria	Small to highly motile rods; G -ve

NCBI National Centre for Biotechnological information (NIH, USA), RDP Ribosomal database project at the Michigan State University, East Lansing, USA, T type strain E-value 0 in all the instances

Table 2 Antibiotic sensitivity assay using gentamycin (GM) and cefazolin (CZ) against the isolates retrieved from previously antibiotic-treated watermelon cultures assessed based on inhibition zone development after 2 days

Antibiotic (mg l ⁻¹)	Response to gentamycin					Response to cefazolin				
	GM01	GM02	GM03	GM04	GM05	CZ01	CZ02	CZ03	CZ04	CZ05
0	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–
50	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	–	+
250	–	–	+	+	++	–	–	–	–	++
500	–	–	+	++	++	–	–	–	–	++
1,000	–	–	+	++	++	++	+	–	–	++
10,000	+++	+++	+	+++	+++	+++	++	–	–	+++

Scoring based on diameter of clear zone: – none, + <10 mm, ++ 10–15 mm, +++ >20 mm

GM01 *Cellulosimicrobium* sp., GM02 *Microbacterium* sp., GM03 *Sphingobacterium* sp., GM04 *Brevundimonas* sp., GM05 *Gordonia* sp., CZ01 *B. pumilus*, CZ02 *Cellulosimicrobium* sp., CZ03 *R. radiobacter*, CZ04 *B. safensis*, CZ05 *Xenophilus* sp

remained still unstained, or their staining was detected only in some planes.

Molecular screening of watermelon tissue-DNA with bacterial primers

PCR employing bacterial universal primers as well as different class-specific primers using genomic DNA from index-negative watermelon stocks, adopting the basic program for the primers 9bfm and 1512uR as per Mühling et al. (2008), proved positive to the universal primers 27F and 1492R and 9bfm and 1912rR besides the specific primers targeting cyanobacteria-chloroplast, β proteobacteria, high G + C firmicutes and *Bacillus* spp. giving bands matching the expected size for the bacterial group (Fig. 3). The groups α and γ proteobacteria and actinobacteria which did not show the amplification in the first round of PCR-yielded product of expected size with the adoption of PCR conditions specified for the group or with the use of the first round PCR product from 9bfm, and 1512R as the template (data not presented).

Attempting to bring non-culturable organisms to cultivation

Tissue homogenate from the index-negative cultures plated on NA and TSA did not show any cultivable organisms but numerous motile bacterial cells were observed during microscopy. Tissue chopped and incubated (1 ml broth in 2 ml microfuge tubes, 160 rpm, 30°C) for up to 10 days in nutrient broth (NB), trypticasein soy broth (TSB) or brain heart infusion broth did not show any bacterial colony growth upon spotting (5 μ l lots) on the respective agar-gelled medium up to 2 weeks. Tissue homogenate (one part) from such index-negative cultures, prepared in WMM broth

(3% sucrose) and mixed with NB or TSB (ten parts) also did not show any colony growth upon spotting after 2–7 days of incubation, but that mixed with 0.1 \times NB or 0.1 \times TSB (1:10 proportion) yielded colony growths upon testing on NA/TSA after 7 days of stationary incubation. Once activated to cultivation from the tissue extract with low levels of nutrition, the organisms showed normal or even better growth on 1 \times medium than on 0.1 \times NA and TSA.

The colony growths formed on NA from the spotting of tissue homogenate in the latter attempt yielded four different colony types upon dilution plating, two each from the 0.1 \times NB and 0.1 \times TSB sources. The resultant organisms were identified as *R. radiobacter*, *B. pumilus*, *B. firmus* and *Micrococcus luteus* (Table 3). Two isolates (*R. radiobacter* and *B. pumilus*) were identical to the organisms retrieved earlier from the ageing stocks.

Host tissue extract assay

Watermelon cultures that were incubated without sub-culturing for extended periods showed obvious bacterial growth emanating from the tissue in senescent stocks after 6–12 months of undisturbed incubation with the culture bottles still remaining sealed in sterile PP bags. Most of the organisms isolated in this study showed a very faint or non-obvious growth on plain WMM (Fig. 4a). On the other hand, they displayed obvious and accelerated growth of most of the organisms on medium supplemented with HTE, which indicated that the host tissue constituents exerted a beneficial effect on the growth of the organisms (Fig. 4b). The quantitative assay using HTE in 0.1 \times NB at 100 μ l ml⁻¹ showed a substantial increase in the growth of all the organisms compared with the respective control (Fig. 5), and this was significant ($P < 0.05$) for all the organisms except *Xenophilus* sp. (CZ05).

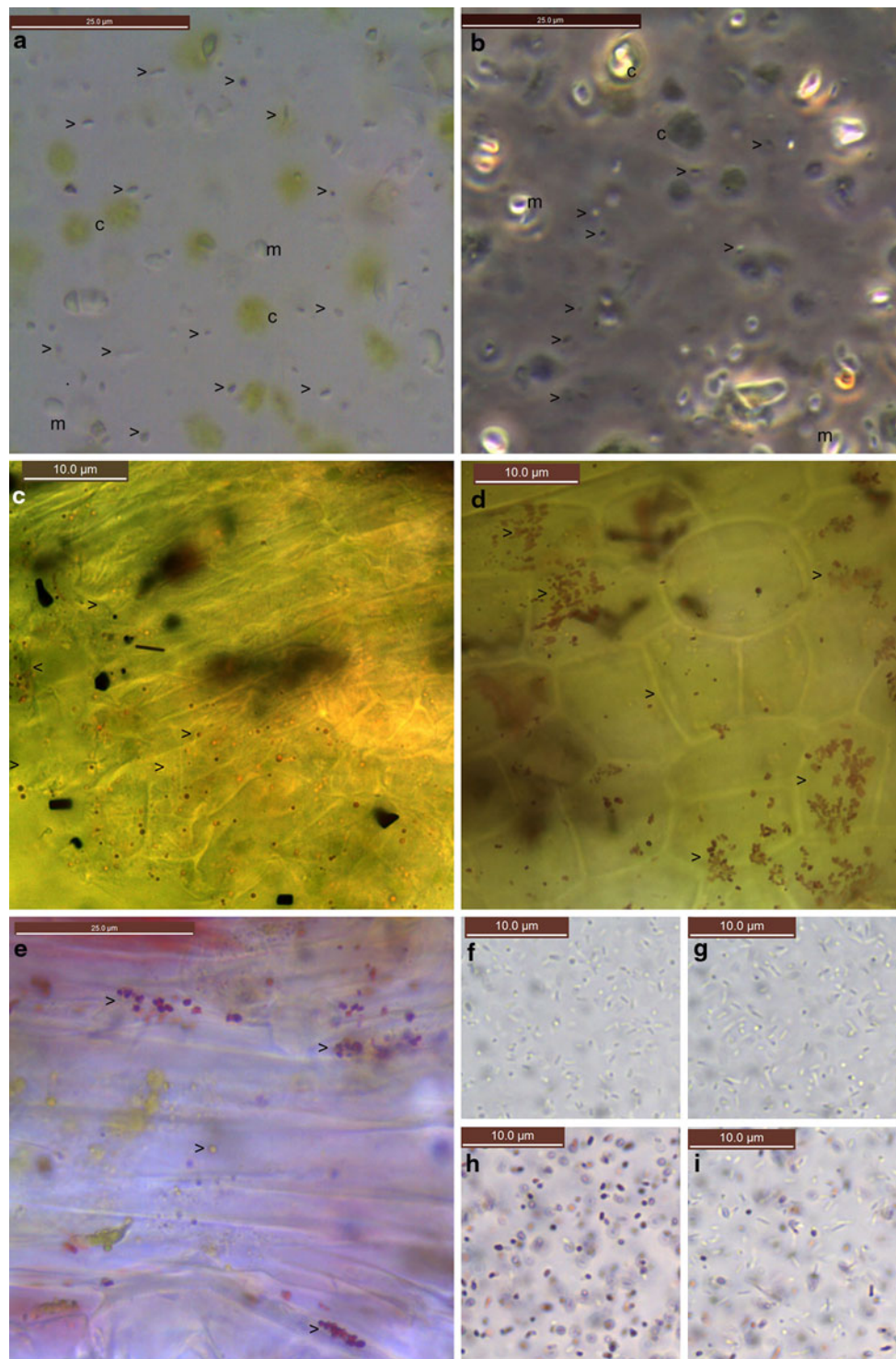


Fig. 2 Microscopy ($\times 100$ objective) on tissue squeeze and tissue sections of healthy and index-negative watermelon cultures showing tissue-associated bacteria. Tissue squeeze under bright field (**a**) and phase contrast (**b**) showing bacterial cells including cocci, oblong and rod shaped cells; free hand tissue sections from shoots incubated in TTC solution after surface sterilization of index-positive tissue (**c**),

obviously contaminated culture after surface sterilization (**d**) after 1 d in TTC, and that from index-negative tissue after 7 days in TTC (**e**) in comparison with pure cultures of bacteria in water film (**f**, **g**), or after overnight TTC treatment (**h**, **i**) under bright field (*arrow heads* indicate bacterial cell; *c* chloroplast, *m* mitochondria, *Bar* 25 μ for **a**, **b**, **e**; 10 μ for others)

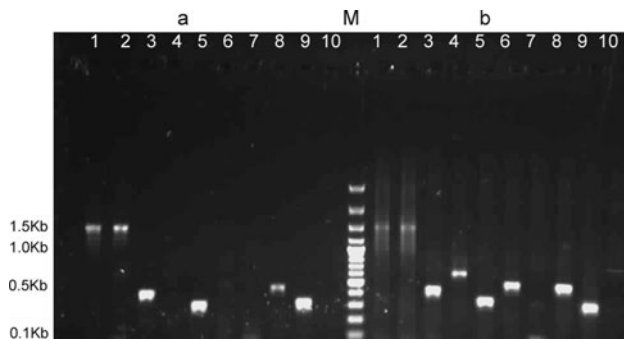


Fig. 3 PCR on whole genomic DNA extracted from index-negative surface sterilized watermelon stocks using universal and bacterial class-specific primers. **a** PCR employing the basic program for the primer pair 9bfm and 1512uR as per Mühling et al. (2008) on watermelon tissue-derived DNA sample and **(b)** using the DNA template from pure bacterial cultures as positive control. *M* 1 Kb ladder; 1–10 indicate primer pairs 27F and 1492R (1), 9bfm and 1512uR (2), CYA 361f and CYA 785R (3), Alf 28f and Alf 684r (4), Beta 359f and Beta 682r (5), Gamma 395f and Gamma 871r (6), Act 243f and Act 1492r (7), Firm 350f and Firm 814r (8), Bacil 47f and Bacil 365r (9) and negative control (10) (alfa and gamma proteobacteria tissue DNA, and Actinobacteria control and tissue DNA samples showed amplification in the second attempt with the adoption of PCR conditions specified for the group, or PCR using the product from 9bfm, and 1512uR as the template)

Discussion

Plants interact with diverse categories of microorganisms in their growing environment, described variously as pathogens, symbionts, rhizobacteria, epiphytes, endophytes, etc. (Bacon et al. 2002; Rosenblueth and Martinez-Romero 2006; Hardoim et al. 2008). On the other hand, in vitro cultures are supposedly alienated or protected from all such organisms. Following the initial realization that some bacteria could survive in tissue cultures with no obvious indications of their presence (Thomas 2004a, b), we took

up cleansing of watermelon culture through antibiotic treatment. The objective was supposedly attained (Thomas et al. 2006) until we encountered another bout of contamination, this time in senescent and degenerating cultures.

The observations with colony growth-displaying watermelon cultures, response of retrieved organisms in antibiotic sensitivity assay, parallel findings with the visibly clean index-negative watermelon cultures, the strict sterility regimes followed during and after the cleansing operation all indicated that the bacterial growth in the degenerating cultures emanated from endophytic organisms that withstood or escaped the previous antibiotic treatment on account of their tolerance to the chemical or the level employed. They went undetected during the recurrent screenings over 2 years on account of their non-culturability on diverse bacteriological media. The organisms gradually multiplied with time after the withdrawal of antibiotics, yet being non-culturable on the media employed. The activation of associated organisms to cultivation in the degenerating cultures was possibly facilitated by the release of tissue breakdown products as suggested in the HTE supplementation assays. HTE-mediated activation of non-culturable organisms has been documented in other systems too (Thomas and Soly 2009).

In this study, microscopic and molecular validation of endophytes was carried out mainly on counterpart healthy cultures that were in active micropropagation and not showing obvious bacterial contamination or culturable bacteria during tissue-indexing. Results from PCR screening on tissue-DNA from surface-sterilized shoots further endorsed that the organisms were internal inhabitants. This also confirmed the presence of organisms belonging to different classes including *Bacillus* sp., which is usually regarded as a laboratory contaminant. The active motility of live bacterial cells in tissue sap or fresh tissue sections

Table 3 Endophytic bacteria activated from shoot tips of index-negative watermelon cultures through tissue-crush enrichment

No.	Isolate ID	16S seq (bp) and NCBI Acc. No.	Closest NCBI species identity and homology	RDP best type strain match	Suggested identification and phylogenetic group	Cell type under phase and Gram reaction
1.	WM-TH01	505 (HM146147)	<i>Agrobacterium tumefaciens</i> (100%; FR828333)	<i>Rhizobium radiobacter</i> (T) IAM 12048 (AB247615; 0.897)	<i>Rhizobium radiobacter</i> ; α proteobacteria	Medium long motile rods, G –ve
2.	WM-TH02	589 (HM146148)	<i>Bacillus pumilus</i> (100%; EU366363)	<i>B. pumilus</i> (T) ATCC 7061 (AY876289I; 0.963)	<i>Bacillus pumilus</i> ; Firmicutes	Rods, spore former, G +ve
3.	WM-TH03	660 (HM146149)	<i>Bacillus firmus</i> (99%; EU418717)	<i>B. firmus</i> (T) IAM 12464 (D16268; 0.884)	<i>Bacillus firmus</i> ; Firmicutes	Rods, spore former, G +ve
4.	WM-TH04	611 (HM146150)	<i>Micrococcus luteus</i> (100%; EU071591)	<i>M. luteus</i> (T) DSM 20030 (AJ536198; 0.987)	<i>Micrococcus luteus</i> ; Actinobacteria	Cocci; G +ve

NCBI National Center for Biotechnological information (NIH, USA), RDP Ribosomal database project at the Michigan State University, East Lansing, USA, *T* type strain

E-value 0 in all the instances

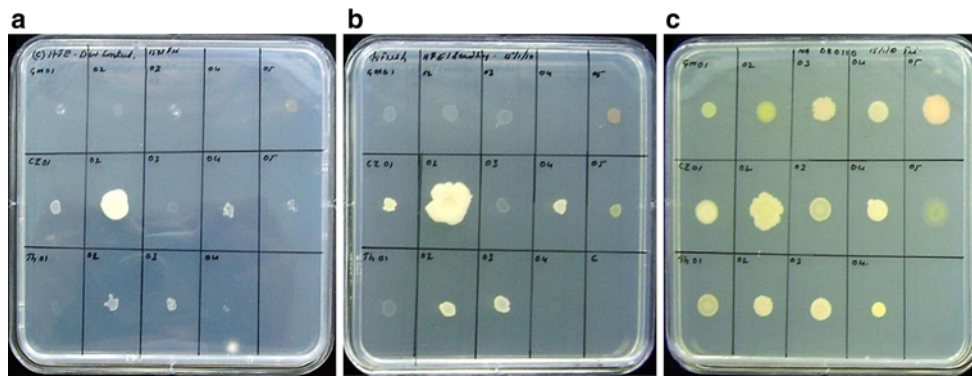
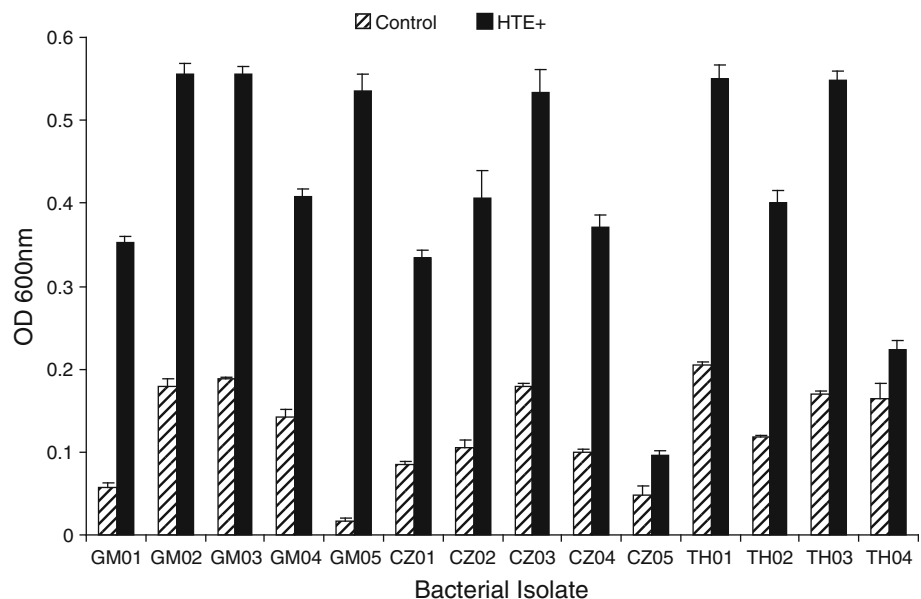


Fig. 4 Effect of tissue homogenate supply on colony growth and expression of bacterial isolates retrieved from watermelon cultures on tissue culture medium. Faint or non-obvious growth on plain WMM (a), enhanced and obvious growth on WMM with HTE supply at

$10 \mu\text{l cm}^{-2}$ (b), and the NA plate used to confirm the presence of inoculum (c). Row 1 isolates GM-01 to 05, row 2 CZ-01 to 05 and row 3 TH-01 to 04 (isolates as per Tables 1, 3)

Fig. 5 Effect of supplementation of $0.1 \times$ nutrient broth with HTE on the growth of bacterial isolates retrieved from watermelon cultures. $0.1 \times$ NB control versus broth incorporated with host tissue extract at $100 \mu\text{l ml}^{-1}$ with the OD (mean \pm SD) determined after 3 days of incubation at 30°C at 220 rpm (isolates as per Tables 1, 3)



captured through videography under bright field or phase contrast and the similarity to the pure bacterial cultures isolated from the same source provided a clear evidence of the internal tissue colonization in healthy watermelon cultures. The wriggling movement of bacteria in the tissue squeeze *prima facie* appeared like Brownian movement but the similarity to that of pure cultures, particularly actinobacteria group and small-celled proteobacteria, endorsed that this was not a mere passive movement. This, however, differed from the expressly active motility observed in some organisms like *Bacillus* spp. and *Rhizobium*. Vital staining technique using TTC which detects dehydrogenase activity in live bacterial cells (Kumar and Tarafdar 2003), also proved to be a valuable simple tool to study endophytes in contrast to exploring them in fixed tissue where

the bacterial cells are not easily discernible from minor organelles and other tissue inclusions (Thomas et al. 2007, 2008). The study suggests direct observations on fresh tissue sections and live microscopy as a simple way to study the endophytes unlike in fixed tissue (Thomas et al. 2007). The exact regions of colonization need to be elucidated now which is next on the agenda.

The present study on watermelon differed from our previous reports on banana and papaya depicting the ubiquitous association of endophytes (Thomas et al. 2007, 2008) in respect of the additional antibiotic treatment provided which was expected to have severed the endophytic association as per the information available at that time (Thomas et al. 2006). Recent studies using tissue cultures have helped in elucidating the widespread

association of culturable and non-culturable endophytes with in vitro cultures, which had their origin from field-derived shoot buds (Pirttilä et al. 2000, 2005; Thomas et al. 2007, 2008; Thomas and Soly 2009). This information on ubiquitous association of endophytes possibly has evaded the wide attention of plant biologists on account of the general non-culturability of associated organisms. The above studies employing banana and papaya have indicated that only a minor fraction of the bacterial cells detected during microscopy on in vitro plant cultures were culturable on common bacteriological media while majority of cells remained as viable but non-culturable (VBNC). VBNC endophytes have been documented in different plant systems (Sun et al. 2007; Bulgari et al. 2009).

The current observations suggested that some bacteria possess the ability to switch between culturable and non-culturable states as per their requirement or the prevailing conditions. Several plant-associated bacteria including Gram-negative symbionts (Alexander et al. 1999) and pathogens (Ordax et al. 2006) are known to enter the VBNC state. This is generally viewed as a survival strategy with the ability to resuscitate upon the return of favorable environment. *Methylobacterium* sp. in potato in vitro cultures turned cultivable once the tissue was infected by another bacterium, *Pseudomonas* sp. (Podolich et al. 2009).

The observations in this study endorsed that the apparently clean plant tissue cultures are not aseptic literally, but only free from hazardous associations, and suggest the need for taking into account such covert and non-culturable endophytes while describing in vitro culture systems and in molecular profiling studies such as for assessing clonal fidelity. Plant associated bacteria are known to produce phytohormones and the same applies to the endophytes isolated in this study (Thomas and Upreti, unpublished results). When a culture is not suspected as contaminated, the activity of associated organisms may appear as normal plant activities and may not be recognized as of microbial in origin (Holland 1997; Holland and Polacco 1994). Such unsuspecting associations may be involved in tissue culture related issues like habituation, epigenetic variation and somaclonal variation as discussed elsewhere (Thomas et al. 2007, 2008).

The growth enhancement of organisms with HTE supplementation suggested the reliance of endophytes on the host in tune with the observations from other plant systems like banana (Thomas and Soly 2009) and papaya (Thomas et al. 2007; Thomas and Kumari 2010). Whereas the supply of HTE or the presence of tissue breakdown products was warranted for the bacterial conversion from non-cultivable to cultivable form, once activated, the organisms grew on different media without the obligate requirement for the host tissue or tissue constituents. The observations also raise the issue about the safety of gene banks with the

potential threat of such organisms getting activated with the extended culture incubation in vitro, or at culture revival at higher incubating temperatures than during the conservation phase (Panicker et al. 2007; Thomas and Kumari 2010). The in vitro cultures often remain unaffected from non-culturable or obligate endophytes but succumb under stress or with the loss of healthy status.

The observations also suggest tissue culture system as a valuable tool to study plant–endophyte interaction under controlled conditions away from the interfering microorganisms unlike in the ambient environment. Most of the organisms isolated in this study like *Cellulosimicrobium*, *Microbacterium*, *Sphingobacterium*, *Brevundimonas*, *Gordonia*, *Xenophilus* and *Micrococcus* spp. are not so common bacteria. Endophytes are considered to be important in agriculture as agents in plant growth promotion, stress alleviation and bioremediation (Ryan et al. 2008; Hardoim et al. 2008; Compant et al. 2010). The ability to retrieve novel organisms through the activation of normally non-culturable organisms making them amenable for the future exploitation is an added advantage with the tissue culture systems.

The triploid watermelon accession used in this study has now completed 15 years in vitro and is now over 5 years after the previously attempted bacteria cleansing. All the cultures now tend to show bacterial growth at the shoot base in case the sub-culturing is delayed beyond 4–6 weeks by when the cultures loose their sheen with the onset of senescence. Now, it warrants a surface sterilization at each sub-culturing after excising the shoot growth above the basal contaminated part to keep the cultures growing actively. Antibiotic treatment is often prescribed to safeguard against bacterial contaminants (Teng and Nicholson 1996; Misra et al. 2010) and for culture cleansing (Tanprasert and Reed 1997; Kulkarni et al. 2007). The observations here suggest that use of antibiotics even with recurrent culture-indexing as had been practiced by us (Thomas and Prakash 2004; Thomas et al. 2006), might offer only a temporary solution to the issue of endophytic association as several organisms show antibiotic tolerance and remain in a VBNC state, thus going undetected on tissue culture media or during the bacteriological media-based tissue screenings. The organisms that survived the treatment would multiply gradually and reappear later. Elimination of all the associated endophytes also seemed undesirable as experienced with our bacteria-cleansed grape culture (Thomas and Prakash 2004), which gradually lost its fitness and survivability in vitro (Thomas, unpublished results).

In conclusion, the present study unravels the source of bacterial growth encountered with a section of supposedly bacteria-cleansed watermelon cultures as the remnants of antibiotic-tolerant non-culturable organisms which

gradually multiplied in them and got activated to cultivable form with culture ageing, possibly mediated by the tissue-breakdown products. The study endorses the intense and ubiquitous association of endophytic bacteria with the host under in vitro conditions in abundant numbers and the utility of tissue culture system to study the host–endophyte association away from unsolicited organisms and to retrieve uncommon endophytes.

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