A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures

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A sequential three-step screening procedure to detect any covert or endophytic bacteria in plant tissue cultures was developed consequent to the observation that conventional detection methods were inconsistent and undependable. This procedure involved diligent visual examination of cultures for any inconspicuous growth (step-1), indexing the medium of visually clean cultures using bacteriological media (step-2), and subsequent tissue-indexing (step-3) using split segments from different plant parts. Step-2 indexing of grape, watermelon, papaya, capsicum, eggplant and gerbera cultures revealed bacteria in 0–100% cultures in different batches. Varying proportions of cultures that passed step-2 indexing turned positive during step-3 indexing, suggesting the essentiality of tissue-indexing. Use of two bacteriological indexing media (BIM), namely nutrient agar (BIM₁) and 523 medium (BIM₂) differing in nutrient constituents, pH (6.4 and 7.0 respectively) and gel strength (10 and 20 g L⁻¹ agar), pre-incubation of nutrient plates at 30–37°C to ensure freedom from incidental contaminants, post-indexing incubation at two different temperatures (25–30°C and 37°C respectively) and sterility testing of tools prior to use were other considerations during indexing. This screening procedure practised for two–four cycles allowed reliable scrutiny of plant tissue cultures for freedom from cultivable bacteria at culture initiation or while sanitizing contaminated cultures; this would find application for certification of in vitro cultures and gene banks.

PLANT tissue culture offers an important tool for rapid clonal multiplication of elite plants, crop improvement, genetic transformation, basic morphogenesis studies, and conservation and exchange of germplasm. To attain these goals, cultures should essentially be free from all microorganisms. Bacteria, which may be introduced in cultures as epiphytes and endophytes or later during culture handling, sometimes remain covert or latent¹⁻³ and go unnoticed in the absence of specific indexing⁴⁻⁵. Presence of covert bacteria in the cultures is highly undesirable due to obvious adverse effects on growth⁶⁻⁷, lack of reproducibility of tissue-culture protocols⁷, possible hormone-mediated growth effects⁸, ramifications in cell cultures⁹, possibility of carrying pathogens¹⁰, potential risk to in vitro gene banks¹¹ and safe exchange of germplasm¹². All these reduce the reliability of plant cell/tissue-culture systems¹³⁻¹⁷.

Availability of a reliable screening method is the primary requirement for tackling the covert contamination problem. Many studies have addressed the isolation and identification of common bacteria in tissue cultures⁵,⁶,¹², but reliable detection which is essential to ascertain asepsis remains neglected. Testing the cultures using bacteriological indexing medium (BIM) is the simplest and best method as it allows non-specific detection of a wide range of bacteria even when present in low numbers²,¹³. Molecular and serological methods allow detection of specific organisms but they are expensive, expertise-demanding and of limited use for general indexing²,¹⁸. Conventional indexing methods include incorporation of bacterial growth-enhancing constituents in the tissue culture medium¹⁵, placing the tissue in enriched liquid medium and assessing its turbidity¹²,¹⁶, or streaking the base of plantlets on BIM¹⁵. Placing pieces of tissue on BIM or testing the tissue homogenate on BIM¹⁷ has been suggested to detect any endophytic bacteria that survived initial decontamination treatment.

While handling long-term micropropagated cultures of seedless watermelon and grape, covert bacteria were found rampant in them, which emerged as the cause of the in vitro decline manifested in the form of poor growth response, severe drop in propagation rate, lack of rooting and root growth in them¹⁷. During the efforts to sanitize the cultures, use of the above-mentioned conventional indexing methods resulted in inconsistent and inconclusive results. Often bacteria resurfaced during subsequent cycles similar to other reports on freeing the cultures from bacteria employing antibiotic treatment⁹,¹⁰. Based on the observations over three years using established as well as fresh cultures of various plant species, a step-wise screening involving visual examination, medium-indexing and tissue-indexing was found necessary for reliable detection of covert and endophytic bacteria. This article outlines the procedure and precautions to be taken during the indexing of plant cultures.

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Materials and methods

The studies were carried out mainly using long-term micropropagated cultures of grape (Vitis vinifera L.) cv. Arka Neelamani\(^2\) and triploid watermelon (Citrullus lanatus Thunb. [Matsum. & Nakai]) cv. Arka Manik\(^2\) during their 5–9-year period in vitro. Grape was raised on a medium gelled with phytagel (Sigma Chemical Co, St. Louis) as described elsewhere\(^2\), which gave a clear medium, while watermelon was raised on a medium gelled with agar (Sigma) owing to hyperhydricity\(^2\) that gave a semi-transparent medium. Both the cultures showed single shoot growth, and shoot-tip and nodal microcuttings from these stocks were used for propagation\(^1,2\). Fresh culture of diploid watermelon (cv. Arka Manik) was initiated from seeds and gerbera (cv. Pink Elegans) from capitulum explants\(^3\), while cultures of papaya (cv. Surya), grape (cv. Thompson Seedless), capsicum (cv. Arka Gaurav) and egg-plant (cv. Arka Keshav) were obtained from colleagues. Grape and watermelon cultures in this study refer to long-term maintained stocks, unless mentioned otherwise.

The cultures were grown in bottles (120 mm height \times 65 mm diameter) with a screw cap providing near air-tight sealing, unless mentioned differently. Grape stock was subcultured at 6–8 weeks interval and watermelon at 4–6 weeks using four microcuttings per vessel in 50 ml medium. All the cultures were incubated at 26 ± 2°C under 16 h photoperiod (30–40 \(\mu\)E m\(^{-2}\) s\(^{-1}\)) provided by cool-white fluorescent tubes.

The optimized screening procedure evolved based on observations from different trials consisted of three steps, including diligent visual examination of cultures (step-1), medium-indexing of visually clean cultures (step-2), and tissue-indexing of medium-index-negative cultures (step-3). The cultures were first observed at eye level, from above and from underneath with and without background light for any inconspicuous bacterial growth. Two BIMs were identified based on the preliminary trials towards medium- and tissue-indexing. These included nutrient agar (NA) containing 5 g l\(^{-1}\) each peptone and NaCl, 3 g l\(^{-1}\) beef extract and 20 g l\(^{-1}\) agar (BIM\(_1\)) and 523 medium of Viss \textit{et al.}\(^4\) containing 10 g l\(^{-1}\) sucrose, 8 g l\(^{-1}\) casein hydrolysate, 4 g l\(^{-1}\) yeast extract, 2 g l\(^{-1}\) KH\(_2\)PO\(_4\), 0.15 g l\(^{-1}\) MgSO\(_4\)\(_7\)H\(_2\)O and 10 g l\(^{-1}\) agar (BIM\(_2\)). Medium-indexing was done by inserting into the culture medium a sterile 200 \(\mu\)l disposable tip attached to a bleach-swabbed pipette (or using a flame-sealed inoculation needle for cultures growing in culture tubes and those with vigorous shoot growth) and bringing the same in contact with the two BIMs. Before tissue-indexing, the forceps were first indexed on BIM to ascertain their sterility with their results known along with tissue-indexing results. In case of micropropagated cultures, shoot part (one plant per vessel) was excised using a pair of sterile scissors. After sub-culturing the microcuttings, left-over tissue segments from upper/middle part and lower half of stem (5–8 mm) were split longitudinally and one segment was placed on each BIM. The stump was lifted and split-segments from basal swollen part and roots (if available) were placed on BIM. Other plantlets in the same vessel were tested using pieces of basal swelling or root after culturing the microcuttings. Finally the medium was indexed to confirm the step-2 screening results. Cultures other than the micropropagated ones were indexed using shoot, root, regenerating tissue and/or callus according to the availability.

Step-1 and step-2 screening was carried out mostly using the long-term cultures of grape and watermelon (40–100 bottles at any given time), while step-3 screening was undertaken using visually clean and medium-index-negative cultures that were subjected to HgCl\(_2\) (0.05 or 0.1%) or sodium hypochlorite (4% available chlorine; Sd-fine Chemicals, Mumbai, India) treatment as discussed later and/or to antibiotics for sanitizing them\(^7,8\). Cultures of other plant species were used for validation of the results. Fresh sterile petri dishes were used for handling each vessel of the culture and the tools were autoclaved before use on any day, and sterilized in a glass-bead sterilizer (250°C, 3–5 min) or over a gas flame (30–40 s) between cultures after wiping-off any adhering medium using ethanol-drenched cotton. Alcohol dip and flaming were shunned owing to the possibility of transmission of some contaminants through flamed tools\(^9\). BIM in sterile single-use plates (10 mm \times 15 mm; Hi-Media, Mumbai, India) was pre-incubated (up-side-down) at 37°C for 2–3 days followed by 2–4 days at room temperature (approx. 25°C) before use to ensure freedom from incidental contaminants and to evaporate away any free water on the surface so as to avoid spreading colony growth.

During medium-indexing, 20–100 cultures were used per batch, accommodating 20–32 cultures per plate and 4–12 cultures during tissue-indexing. Post-indexing, BIM\(_1\) plates were incubated in the dark at 37°C and BIM\(_2\) at 25 or 30°C for 2–7 days upside-down followed by another 3–4 weeks at room temperature. The plates were observed on days 1, 2, 3 and 4 and thereafter weekly to detect any slow-growing bacteria. The cultures were classified as ‘index-positive’ based on visible bacterial growth from one or more indexed spots or tissue samples on one or more BIMs. Application of statistical treatments did not appear pertinent because of the unique situation in a study of this kind.

Results

Any culture showing obvious microbial growth in the medium could easily be picked up and discarded, and this was encountered to the tune of 0–5% in different batches. Visibly clean cultures showing any faint growth on the medium surface or ‘halo’ at the base of plantlets could be picked up if examined carefully before the roots grew extensively. Occasionally hazy streaks delusive of preci-
pitated medium constituents were seen within the gelled medium and such cultures often tested positive during medium-indexing. Dry grainy tracts on the surface which mimicked dried-up water/medium turned out to be faint bacterial patches on the surface when observed from below. Loss of clarity of medium, general pale growth, partial drying of leaves (Figure 1a) and emission of a foul odour upon opening the vessels were other indicators of covert bacteria. Thus, a certain proportion of covertly contaminated cultures could be identified based on diligent visual examination. Indexing of medium, however, made it easier to detect unambiguously such cultures.

Medium-indexing revealed covert bacteria in the range of 0–100% in various plant cultures with consistent results. Use of disposable tips or inoculation needle gave confined colony growth around the point of inoculation, unlike with explant base-streaking\(^4\) and accommodated several cultures per plate (Figure 1b). Testing the culture medium at several points and spotting at 4–5 points on the BIM using the same tip offered better or faster contaminant detection. The medium collected in the pipette tips also formed a good sample for indexing, but it demanded careful dispensing onto the BIM to avoid its spattering and scattered colony growth. Provision of a 2 ml overlay of half-strength liquid BIM in the cultures a week before and testing 1–2 μl of this medium allowed detection of bacteria that were not evenly spread on the medium.

In the preliminary trial where BIM\(_1\) and BIM\(_2\) were tested with other BIMs which included NA with 10 g L\(^{-1}\) sucrose (BIM\(_3\)), half-strength Murashige and Skoog medium\(^{27}\) with 10 g L\(^{-1}\) each sucrose and dextrose, 8 g L\(^{-1}\) casein hydrolysate, 4 g L\(^{-1}\) yeast extract, 2 g L\(^{-1}\) peptone (BIM\(_4\)), MS constituents in BIM\(_4\) substituted with half-strength Rugini medium\(^{28}\) (BIM\(_5\)), 4 g L\(^{-1}\) each casein hydrolysate and peptone, 8 g L\(^{-1}\) NaCl and 10 g L\(^{-1}\) dextrose (BIM\(_6\)), casein hydrolysate in BIM\(_6\) substituted with beef extract (BIM\(_7\)), BIM\(_7\) supplemented with fresh extract from 10 g L\(^{-1}\) potato (BIM\(_8\)), Luria broth agar (BIM\(_9\)), potato dextrose agar (BIM\(_{10}\)) and BIM\(_{10}\) containing 10 g L\(^{-1}\) dextrose (BIM\(_{11}\)), the extent of detection and speed of colony growth differed with various BIMs (data not presented). Use of potato extract in the medium did not contribute to better or faster detection. Use of dextrose, higher pH (7.0) and low gel strength in general favoured earlier and faster colony growth, but lower pH (6.4 or 5.8) and stronger gelling offered confined colonies and clearer results. Similarly, incubation of plates at 37°C enhanced the speed and rate of colony growth, while overall detection after one week was relatively better at 25–30°C (data not presented).

Tissue-indexing of visually clean and medium-index-negative cultures showed bacterial growth on one or both media within 2–4 days in 0–100% of the cultures depending on the pre-treatments given or the extent of screening at steps 1 and 2. Medium-embedded basal swelling which formed a part of the original microcutting and root showed more frequent and early bacterial growth. Some specific examples are presented below to demonstrate covert bacterial survival in the tissue without detectable presence in the medium.

Medium-indexing of a batch of 16 grape cultures four weeks after HgCl\(_2\) treatment (0.1%; 5 min) showed six of them as index-negative on three BIMs, i.e. BIM\(_{12,3}\). Tissue-indexing a week later revealed bacteria in all the indexed tissue parts of one culture (17%), while the medium still tested negative (Table 1). However, the medium turned index-positive in the next passage indicating gradual inoculum build-up with time. The other five cultures remained index-negative for the next three cycles, confirming freedom from cultivable bacteria.

In an experiment aimed at cleansing watermelon cultures through NaOCl treatment (5 min), six of 16 apparently clean cultures on multiplication medium tested positive when indexed on four different BIMs four weeks after the previous culturing (Figure 2a). Tissue-indexing a week later using BIM\(_{12}\) revealed bacteria in the upper or lower stem segments in just one or both media, while the basal segment did not show any bacterial growth on either medium (Figure 2b). This indicated that covert bacteria would not always be brought out through streak test and the need for using more than one indexing medium.

In another study on sanitizing watermelon cultures, ten out of 20 shoot tips that were treated with HgCl\(_2\) (0.05%; 10 min) and planted singly on filter paper bridges in liquid medium (Figure 3a) showed low to moderate turbidity of medium 2 weeks later. Upon testing the ten visibly clean cultures along with eight cultures with low visual turbidity by transferring 2 μl liquid medium, six of the former (60%) and whole of the latter revealed bacteria in them (Figure 3b(a)). Next 100 μl of medium from the four index-negative cultures was plated on BIM\(_1\); at 3 and again 4 weeks from the original culturing which revealed no bacteria in the medium (Figure 3b(b)). When these cultures were subjected to tissue-indexing a week later, endogenous bacteria were found in all four of them (Figure 3b(c)), while the medium remained index-negative.

Eggplant cotyledon and watermelon shoot-tip cultures initiated from aseptically raised seedlings tested index-negative during medium-indexing at 4 weeks after culture.
Table 1. Screening of micropropagated grape cultures for covert and endophytic bacteria planted after treatment with HgCl₂ (0.1%) for 5 min

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Index + ve/− ve and BIM</th>
<th>Step-2: Indexing of culture medium (4 weeks)*</th>
<th>Step-3: Tissue-indexing with sterility testing of forceps (Using BIM1,2,3) (5 weeks)*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>− ve</td>
<td>− ve</td>
<td>− ve</td>
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<tr>
<td>2</td>
<td>− ve</td>
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<td>3</td>
<td>+ ve (BIM1,2,3)</td>
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<td>4</td>
<td>− ve</td>
<td>ND</td>
<td>− ve</td>
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<td>5</td>
<td>− ve</td>
<td>ND</td>
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<td>− ve (BIM1,3)</td>
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<td>− ve</td>
</tr>
<tr>
<td>16</td>
<td>+ ve (BIM1,3)</td>
<td>ND</td>
<td>− ve</td>
</tr>
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</table>

*Time in weeks after decontamination treatment; † Cultures that remained index-negative at all steps. BIM1, NA; BIM2, 523 medium; ‡ BIM3, NA with 10 g L⁻¹ each agar and sucrose. + ve, Index positive; − ve, Index negative; ND, Indexing not done; d₁, Days taken for visible bacterial growth on BIM.

![Figure 2](imageurl) Figure 2. Indexing the medium of visibly clean watermelon cultures on two modified BIMs using disposable tips (a) and tissue-indexing of ‘medium-index-negative’ cultures (1D no. 1, 2, 3, 7, 8, 9, 13, 14, 15 and 16) using BIM1 and BIM2 (b). Sterility testing of forceps; row 2. Stem from upper part; row 3, Lower stem and row 4, Basal swelling). Arrowhead indicates bacterial growth from tissue.

![Figure 3](imageurl) Figure 3. a. Watermelon shoot tips cultured on filter paper bridges showing clear medium as in control tubes. b. (a) Indexing shoot tip cultures by spotting 2 µl medium on BIM1; (b) testing of ‘index-negative’ culture by plating 100 µl medium, and (c) tissue-indexing of four index-negative cultures using split stem segments from upper (row 2), middle (row 3) or lower part of the shoot (row 4). Forceps before use were indexed in row 1.

initiation but showed 0–10% index-positives in different batches during tissue-indexing a week later. Eleven per cent of a batch of 18 visibly clean papaya cultures turned index-positive for medium and 19% of the remaining cultures tested positive during tissue-indexing. In grapes, 17% of the 18 visibly clean new cultures tested both medium- and tissue-index positive. In gerbera, 10 and 25% of the capitulum cultures (80 nos) tested index-positive for medium and tissue respectively, in the first cycle.

In a trial where capsicum cotyledon cultures growing on medium supplied with cefotaxime (500 ppm) and kanamycin (75 ppm) were subjected to medium-indexing, 25% of the 16 cultures were index-positive, which took as much as 4 weeks for visible growth in some instances. However, only 50% of these cultures tested positive during tissue-indexing.

**Discussion**

The present study confirms earlier reports on the possible survival of bacteria in covert or endophytic form in plant tissue cultures1,2,6,9,17 and demonstrates the essentiality of a sequential screening of cultures involving visual examination, indexing of medium followed by tissue-indexing to detect such bacteria. Four types of bacterial contamination/association were found to be prevalent in tissue cultures. This included obviously visible growth which could easily be picked up, inconspicuous growth which might be identified after careful visual examination, covert bacteria in the medium which might be brought out through medium-indexing and endophytic bacteria detection of which needed indexing of tissue. The last three types may
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go undetected and gradually spread to more cultures, contributing to culture degeneration.

Phytigel-gelled medium facilitated better visual detection of inconspicuous bacterial growth compared with cloudy agar-gelled medium, but also allowed accumulation of some bacteria that were not visible in such medium. Eight or more distinct bacterial types were found associated with agar-gelled watermelon medium compared with about six types isolated from phytigel-gelled grape medium. On the other hand, some bacteria (e.g. Bacillus pumilus) showed visible growth on the former medium but survived in subdued form on the latter. This indicated that the transparent medium would not ensure visual detection of bacterial contaminants and the omnipresence of endophytic bacteria which may get easily introduced in the cultures.

Various bacterial species differ in their ability to grow on a particular medium and no single bacteriological medium is able to detect all the contaminants. On the other hand, economic considerations and operational feasibility do not permit the use of a wide array of media or incubating conditions. Thus, use of two indexing media differing in major constituents, pH and gel strength, and two different incubating temperatures was found necessary during step-2 and step-3 indexing. Our choice for general detection of contaminants prevalent under tropical and sub-tropical environments included BIM₁ (pH 7.0; 20 g l⁻¹ agar) with 37°C incubation and BIM₂ (pH 6.4; 10 g l⁻¹ agar) with 25 or 30°C incubation.

Indexing of medium is preferably done 1–2 weeks prior to the intended date of sub-culturing, while tissue-indexing is best done at sub-culturing. Most of the contaminants (> 90%) showed up on BIM within 2–7 days, while some showed delayed growth. Use of sterile disposable tips for medium-indexing avoided the chances of cross-contamination and simplified the operation. The tips could be recycled after autoclaving twice, the first one submerged in water and the second after arranging them in tip-boxes.

The original explant that comes in direct contact with the medium formed the best candidate for tissue indexing in general. Splitting the stem/root facilitates direct contact of endophytic bacteria with the BIM. Distribution of endophytes may be uneven as observed in this study too, and it is desirable to use representative tissue from as many different parts of plantlets as possible. Detection of bacteria in some plant parts following chemical or antibiotic treatment indicated cells trapped or surviving in isolated pockets which would multiply slowly and appear during subsequent cycles. Testing of tissue from various parts facilitated the early detection of such bacteria.

Tissue-indexing should be carried out for the whole lot of primary cultures or chemical/antibiotic treated cultures. Down the line, a small proportion of cultures may be kept as certified stocks, indexed regularly and used to supplement the routine production cultures. Tissue-indexing is more tedious and time-consuming than medium-indexing.

It needs proper record-keeping to track individual cultures. While step-1 and step-2 screening would suffice for general production cultures in a commercial set-up, step-3 indexing is essential for certifying stocks and while cleansing the cultures. Plating the tissue homogenate on BIM for detecting endophytic bacteria is not feasible for regular indexing besides the danger of introduction of contaminants during handling.

In the case of antibiotic-treated cultures, tissue-indexing could give false results due to the residual effect of chemicals imbibed by the tissue and bacteria may reappear months later because of the transient bacteriostatic activity of antibiotics. In such instances, indexing for two- to four cycles after withdrawing the antibiotics would be needed. It may be noted that one surviving cell or spore is enough to cause bacterial reemergence later. Besides ensuring freedom from cultivable bacteria, this indexing approach also helped in detecting any lateral entry of contaminants and in differentiating them from residual endophytes. In this study, we were only concerned about detection of bacterial contaminants but not with the effect of covert bacteria on the growth which can be found elsewhere, or their isolation and identification. The grape and watermelon cultures have now been sanitized of covert and endophytic bacteria through chemical and antibiotic treatments, and this screening procedure has been instrumental to attain this goal.

The screening procedure described here would help in ensuring the safety and reliability of in vitro gene banks, exchange of clean germplasm, checking the multiplication of pathogenic bacteria-harbouthing cultures and in preventing the escape of Agrobacterium-mediated vector systems to the environment. Bacteria harbouring apparently clean cultures shipped across sometimes show up contamination by the time they reach the destination due to fluctuating temperature conditions during transit (personal experience), affecting the supply and reputation of the supplier besides inviting legal issues. The BIMs identified in this study will detect most of the cultivable bacteria but probably not the ones which have specific growth requirements or those that are non-amenable to culturing, detection of which needs molecular-based approach.

It is suggested that the term 'covert bacteria' as described by Holland and Polacco or Horsch and King is more appropriate than the frequently used term 'latent bacteria' to describe such bacteria which are not normally visible on tissue culture medium. 'Latent' is a common term used in plant pathology to describe infections with no obvious adverse effects. Bacterial contaminants in tissue cultures may be latent to the extent that they are not normally visible on tissue culture medium but their effects on plant growth — whether inhibitory, null or promotive — are not clearly known. Thus it has a different meaning from its usage in plant pathology.

In conclusion, plant tissue cultures could harbour bacteria in a totally unsuspecting manner, either externally in

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the medium/plant or endophytically. A sequential three-step screening involving visual examination of cultures, indexing of medium and indexing of tissue from various parts of the culture using two BIMs and elevated incubation temperature is suggested for reliable detection of covert and endophytic bacteria-harbouring cultures and to ensure freedom from such bacteria.


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