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Research Article

Comprehensive Characterization of Bacteria Contaminating Plant Tissue Culture through Morphological, Biochemical, and Antibiotic Sensitivity Analysis

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ABSTRACT

Contamination by bacteria is a common complication and serious reason for difficulties in plant tissue culture. The most crucial factor in plant tissue culture technique is the maintenance of aseptic conditions. However, identification and characterization of contaminated bacteria provide information on the sources of contaminants and therefore limit the problem of contamination. In this study five different contaminated vials of plant tissue culture, such as aloe vera (SP1), orchid (SP2), banana (SP3), wheat (SP4), and rice (SP5) were randomly selected and cultured on the nutrient agar media. Five contaminated samples from two individual colonies were isolated and purified by spread and several time streaking techniques. Based on different cultural, morphological, and standard biochemical characteristics, identification and characterization were done. According to Bergey's Manual of Systematic Bacteriology, the isolates were compared with known organisms and assigned to genera. The all ten isolates were performed to sensitivity tests with three antibiotics. Among ten contaminants isolated from the contaminated plant tissue cultures, most likely four of them were *Bacillus* spp., four of them were *Staphylococcus* spp., and two of them were *Pseudomonas* spp. These findings can support effective contamination management and enhance the success rate of plant tissue culture techniques.

Introduction

Tissue culture is one of the leading tools of plant biotechnology. It has been widely utilized to meet the increasing demand for best planting materials, enabling pathogen-free and healthy plants production throughout the year in reduced time and space (El-Banna *et al.,* 2021). Micropropagation also plays an important role in conserving plant genetic resources (Romadanova *et al.,* 2022).

However, bacterial contamination remains a major constraint in the systems of commercial micropropagation (Kaluzna *et al.*, 2013). Such contamination reduces multiplication, culture initiation, and rooting proficiency, causes tissue necrosis, and may ultimately result in culture mortality (Nadha *et al.*, 2012). Bacterial contamination in plant tissue culture may arise from poor laboratory conditions, inadequate aseptic techniques, or infected explants, with commonly reported genera including *Corynebacterium, Mycobacterium, Rhodopseudomonas, Pseudomonas,* and *Microbacterium* (Romadanova *et al.,* 2022). Assured in vitro conservation and germplasm exchange is essential for eliminating such bacterial infections (Izarra *et al.,* 2020). Treatments of standard sterilization technique such as Tween-20 (10%), ethanol (70%), mercuric chloride (5%), or distilled water are often not enough to demolish these microorganisms (Khan *et al.,* 2018). Antibiotics have been widely used in plant tissue culture to control bacterial contaminants, (Fang & Hsu, 2012; Misra *et al.,* 2010; Shehata *et al.,* 2010). Against in vitro phytopathogens aminoglycoside antibiotics, in particular have shown aptness (Fang & Hsu, 2012). However, antibiotic application must be carefully optimized for each plant species. Because excessive concentrations may induce resistance and cause phytotoxic effects. Therefore, appropriate concentrations should be determined empirically to ensure both efficacy and safety (Thomas *et al.,* 2011;

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Shehata *et al.,* 2010; Shen *et al.,* 2010; Lata *et al.,* 2006; Mirza MS *et al.,* 2001).

These bacterial contaminants can be either endophytic or epiphytic in nature. Epiphytic bacteria colonize surfaces of plants and generally can be destroyed by chemical disinfectants (Hirano & Upper, 1990). But endophytic bacteria live within intercellular spaces of plant tissues (Gunson & Spencer-Phillips, 1994) and using only surface sterilization are difficult to remove (Leifert & Cassells, 2001; Reed *et al*., 1995; Attree & Sheffield, 1986). Contamination of endophyte is particularly problematic as it, rooting rates, growth of plant, and sometimes can cause plant death (Leifert *et al.*, 1991).

Under *in vitro* conditions, by non-pathogenic bacteria plants are physiologically stressed and in this way sometimes become more susceptible to infection (El-Banna *et al*., 2021). Endophytic contamination poses a huge challenge whereas these microorganisms can survive in surface sterilization (Spencer-phillips, 1994). Furthermore, due to unfavorable conditions for microbial proliferation latent bacterial infections may remain undetected, such as low temperatures, unsuitable pH, or high salt concentrations. However, these latent microbes may multiply and impair culture development when conditions shift during plant growth (Izarra *et al*., 2020). In some cases, until cultures are subjected to stress, contamination may not be visually apparent, such as cryopreservation, which can trigger outbreaks of endophytes (Romadanova *et al.*, 2022). A mass range of bacterial endophytes have been reported in micropropagated plants, with commonly identified genera including *Pantoea, Curtobacterium* and *Bacillus.*

Therefore, this study was undertaken to characterize bacterial contamination in plant tissue culture in order to develop effective contamination control strategies. Bacterial isolates were identified through a stepwise approach involving cultural and colony characteristics (e.g., color and colony morphology), microscopic examination (Gram staining and cell shape), and standard biochemical tests (catalase, oxidase, urease, indole production, gelatin hydrolysis, and H_2S production). In addition, antibiotic susceptibility profiling was performed to determine the sensitivity or resistance patterns of the isolates. The identification of contaminating bacteria and their antibiotic response profiles provides a basis for selecting appropriate antimicrobial treatments and optimizing culture conditions, thereby enabling more targeted and effective prevention of contamination in plant tissue culture systems.

Materials and Methods

Collection and preparation of isolates

The contaminated plant tissues including as aloe vera, orchid, banana, wheat and rice were collected from the Tissue Culture Laboratory, Institute of Biotechnology, Bangladesh Agricultural University, Mymensingh. They were named as SP1, SP2, SP3, SP4, and SP5 respectively.

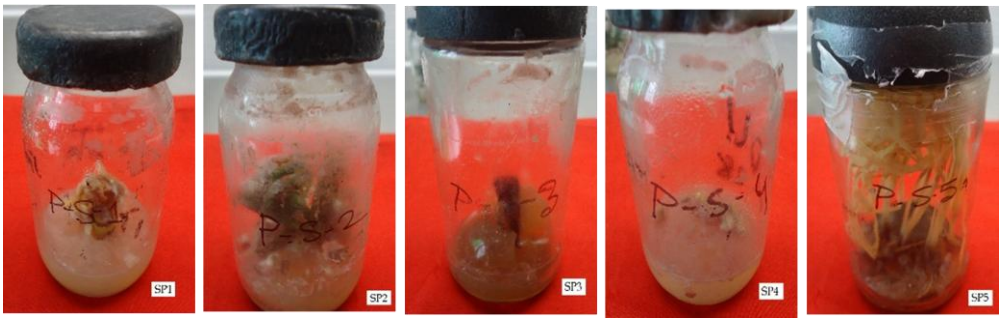

Figure 1: Sample from different contaminated tissue culture vials

At first contaminated tissues were collected aseptically and mashed with mortar. Then 1gm pasted sample was introduced into 9ml 1% peptone water (10^{-1}). Serial dilution was performed until it produced a 10-fold serial dilution. At-last the solution was diluted from 10-fold to 10^{-2}- 10^{-9} serial dilution. For culture, 100 µl diluted sample from each test tube was transferred to the center of a nutrient agar media (NA) petri plate and spreaded using a glass spreader and incubated at 37ºC for overnight followed by (Sathiah & Pan, 2023).

Pure culture preparation

For getting pure colony, streaking method was done on nutrient agar (NA) media and incubated at 37ºC for overnight. Two different isolates from each of the previous plate were used which designated as SP1-C1 and C2, SP2-C1 and C2, SP3-C1 and C2, SP4-C1 and C2 and finally SP5-C1 and C2.

Morphological characterization

In order to determine morphological characteristics of bacterial cells, the cultures were examined

microscopically. The cell shape was recorded as rod or coccus, and gram reaction was positive or negative.

Using streak plate technique, the bacterial isolates were characterized on nutrient agar media and incubated at 37°C for 24 hours and then the colonies were observed for the following characteristics:

i) Shape: Isolated colonies were found as circular or irregular or punctiform.

ii) Color: White creamy, off white, white, orange, and golden yellow colors of colony were found.

Gram's Staining

Gram staining was performed by following the method of (Tripathi & Sapra, 2023). Isolate was smeared on the sterile glass slide and heat-fixed, then stained using crystal violet for 1 minute. Then crystal violet stain was removed using distilled water and stained by iodine. The crystal violet-iodine complex (VIC) was washed using pure ethanol to remove the purple color. And finally counterstained by some drop of safranin and washed by distilled water. The stained-glass slides then placed on a light microscope with a drop of immersion oil (100×), and observed the cell morphologically and the color of the bacteria.

Biochemical characterization

Biochemical tests including Catalase test, Casein hydrolysis test, Starch hydrolysis test, Carbohydrate fermentation tes,t and Indole tests were performed for identification of pathogens. Bacteria were identified according to Bergey's Manual of Systematic Bacteriology (Sathiah & Pan, 2023). From nutrient agar media, colonies were picked up and smeared on the clean glass slide. Then a few drops of 3% hydrogen peroxide were added and bubble formation was observed. The presence of bubbles indicates the catalase positive result and the absence of bubbles indicates negative catalase result explained by (Arbab *et al.,* 2021).

Oxidase test

Presence of cytochrome-c oxidase was tested by Kovac's oxidase test with 1% tetramethyl-p-phenylenediamine dihydrochloride reagent. Bacterial colonies were touched onto reagent-soaked Whatman filter paper using a sterile toothpick. Development of purple or blue color within 5–10 seconds indicated positive oxidase reaction explained by (Garrity *et al.,* 2004).

Urease test

For urease test urea agar slants were prepared and sterilized by autoclaving, then isolates were inoculated at 37°C for 24–48 hours. Development of a deep pink color indicates positive urease test as described by AL-Erjan (2025).

Indole production test

1% Tryptone broth was prepared and sterilized by autoclaving at 121°C for 15 minutes. Test isolates were inoculated and an uninoculated tube was used as a control. After incubation at 37°C for 48 hours, added 1 ml of Kovac's reagent. Tubes were gently shaken and left to stand, allowing the reagent to rise to the top. Result was evaluated by the procedure of Kumar *et al.,* (2024). Formation of a cherry-red layer indicates a positive indole, while no color change indicates negative result explained by (Kumar *et al.,* 2024).

Gelatin hydrolysis Test

Gelatin media was prepared and sterilized by autoclave at 121°C for 15 minutes and 15psi. Using an inoculating loop stab inoculation was performed, from each culture into its properly labeled tubes the loop fool isolates were deep of nutrient gelatin media. The tubes were incubated at 37ºC for 7 days. After 7 days of incubation, the tubes were placed for 30 minutes in refrigerator at 4°C. Then the tubes were observed for liquefaction (Sathiah & Pan, 2023).

Hydrogen Sulphide production test

SIM agar medium was prepared and sterilized using autoclave at 121°C for 15 minutes and 15psi. SIM agar tubes were labeled and each organism was inoculated appropriately. The tubes were incubated at 35° to 36°C for 48 hours. The tubes were observed for the presence or absence of black color along the stab line inoculation followed by (Garrity *et al.,* 2004).

Determination of antimicrobial sensitivity

The susceptibility of bacteria from plant tissue culture was evaluated using the Kirby-Bauer disc diffusion method (Arbab *et al.,* 2021). Bacterial suspensions were prepared and spread on nutrient agar plates using sterile glass spreader and antibiotic discs were applied aseptically. After 24 hours of incubation at 37°C, around the discs the clear halo zones indicated sensitivity. Based on standard guidelines the results were interpreted as sensitive, intermediate, or resistant. Disc concentration (µg) with antimicrobial agents were used as Kanamycin (K)=30(µg). Azithromycin (Azm)=30(µg). Ampicillin (AMP)=25(µg).

Results and Discussion

Contamination by bacteria is a major concern because it hampers the growth and micropropagation efficiency in the plant tissue culture. For developing effective contamination management strategies, it is crucial to understand the nature of these contaminants.

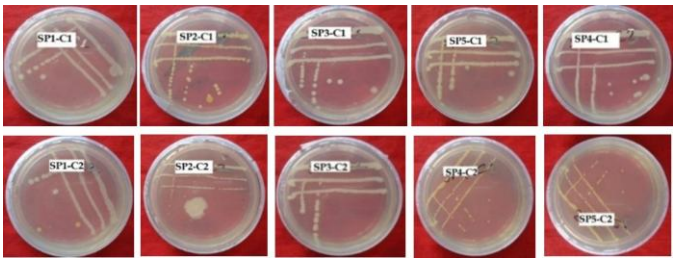

Figure 2: Pure culture of isolates from tissue culture vials

In the present study, from five contaminated plant tissue culture vials, a total of ten bacterial isolates were obtained, including orchid (SP2-C1, SP2-C2), wheat (SP4-C1, SP4-C2), aloe vera (SP1-C1, SP1-C2), banana (SP3-C1, SP3-C2), and rice (SP5-C1, SP5-C2). According to cultural characterization most of the isolates formed circular colonies with white creamy pigmentation; however, SP2-C1, SP2-C2, SP4-C2, and SP5-C2 developed golden yellow colonies (Fig 2 and Table 1). The different colony shape and color are probably due to features that are unique to each species and how they adapt to the tissue culture environment, as seen in other research (Romadanova *et al.,* 2022). In *in vitro* plant cultures the predominance of circular colonies aligns with the general characteristics of saprophytic and endophytic bacteria commonly encountered (Thomas *et al.,* 2017).

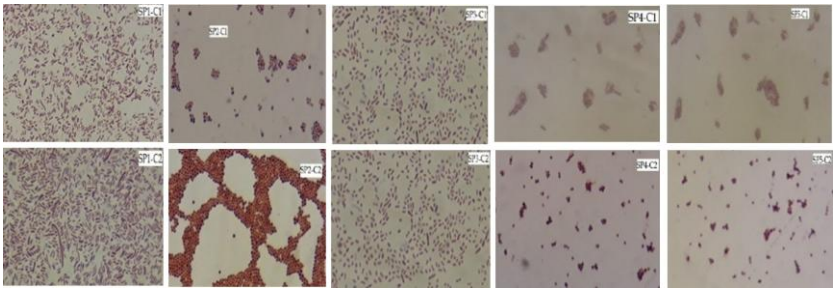

Figure 3: Gram staining of selected isolates

Gram staining morphological analysis demonstrated that the maximum isolates were rod-shaped, with the exception of SP2-C2, SP5-C2, SP2-C1, and SP4-C2 which were coccus-shaped. Gram reaction analysis demonstrated that all the isolates were gram-positive except SP4-C1 and SP5-C1, which were gram-negative shown in Table 1 and Fig 3. The presence of both gram-negative and gram-positive bacteria is consistent with previous reports highlighting the mixed of bacterial flora in the contaminated tissue cultures (Leifert & Cassells, 2001). Gram-positive rod-shaped isolates were indicators of *Bacillus* species (Costa *et al.,* 2024), and gram-positive coccus-shaped isolates assemble to *Staphylococcus* species (Lawson et *al.,* 2006), declaring an origin of endophytic or epiphytic. These findings indicate that in plant tissue cultures contamination is often heterogeneous, involving with differing morphological traits of multiple bacterial taxa *(*Lawson *et al*., 2006).

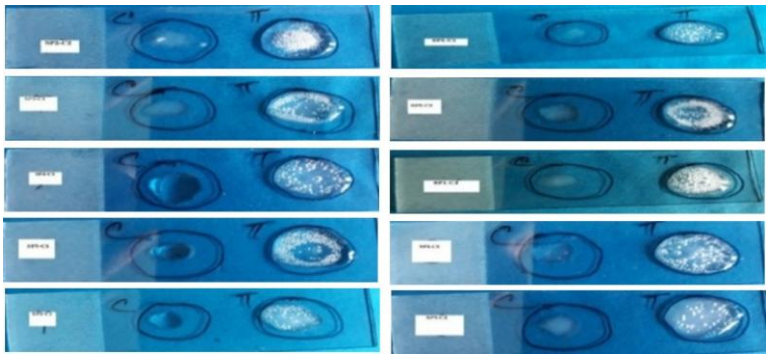

Figure 4: Catalase test of selected isolates

Biochemical characterization yielded more information regarding the isolate's metabolic functions. Bubble formation indicates catalase activity of the isolates. All of isolates showed positive catalase activity presented in Fig 4. This indicates the ability to detoxify hydrogen peroxide, a common oxidative stress defense (Lawson *et al.,* 2006).

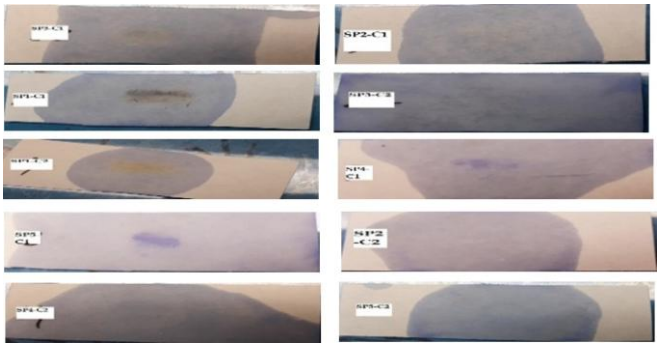

Figure 5: Oxidase test of selected Isolates

The oxidase test showed that SP4-C1 and SP5-C1 were oxidase-positive, it indicates capability of aerobic respiratory, all of oxidase-positive bacteria are normally aerobic and can utilize oxygenase enzyme a terminal electron acceptor in respiration (Hassen *et al.,* 2022) whereas the other isolates were oxidase-negative presented in Fig 5. The urease activity was detected in SP2-C2, SP2-C1, SP5-C2, and SP4-C2 indicates the ability to hydrolyze urea into ammonia (Fig 6) which may influence the nitrogen availability in the culture medium (Wang *et al.,* 2018).

Figure 6: Urease activity test of isolates

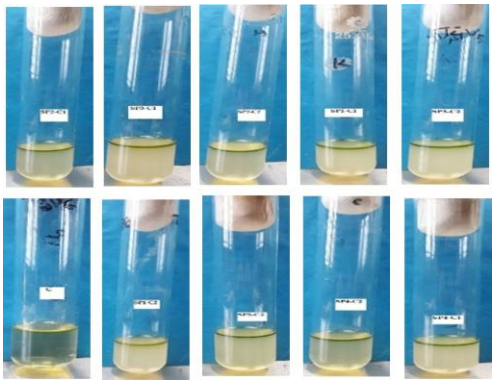

Figure 7: Indole production test

There is no ability to Indole production of all isolates (Fig 7), indicating lack of tryptophanase activity. Noticeably, for all isolates gelatin hydrolysis was positive (Fig 8), it indicates extracellular proteolytic activity (Prigge & Melchinger, 2012), while production of hydrogen sulfide was absent (Fig 9). These biochemical characteristics assemble with the expected profiles of *Staphylococcus, Pseudomonas* and *Bacillus* species (Table 1) (George M. Garrity, 2009). Collectively, the biochemical and morphological profiles confirm the preliminary identification of the isolates and highlight the metabolic diversity among bacterial contaminants.

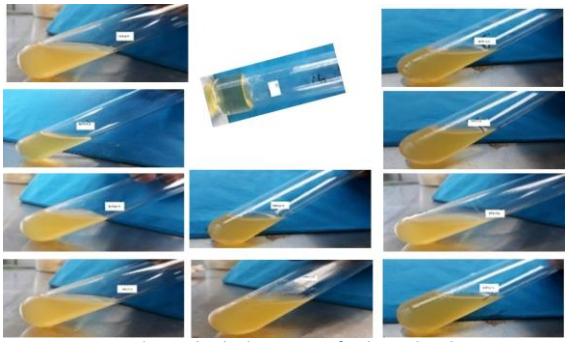

Figure 8: Gelatine hydrolysis test of selected isolates

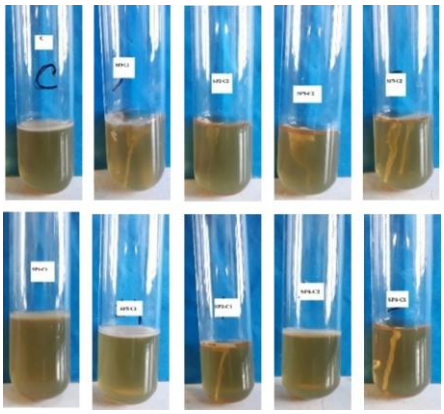

Figure 9: Hydrogen sulphide production test

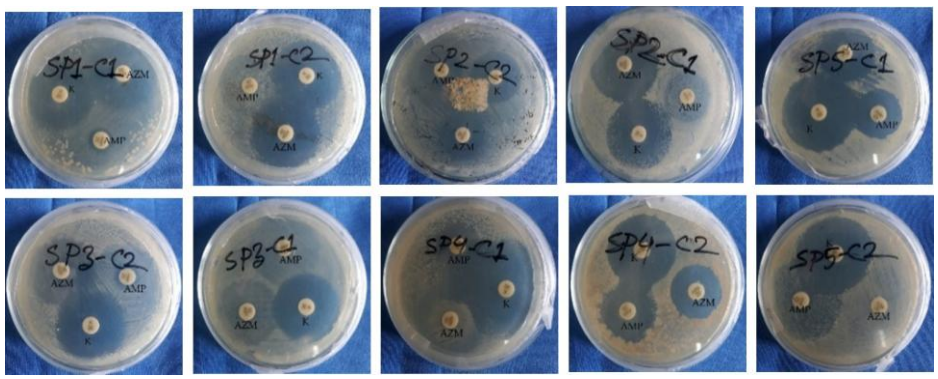

Figure 10: Susceptibility pattern of isolated bacteria against three different antibiotic discs. Note: K= Kanamycin, AMP= Ampicillin, AZM= Azithromycin, SP1-C1, SP1-C2 from aloe vera, SP2-C1, SP2-C2 from orchid, SP3-C1, SP3-C2 from banana, SP4-C1, SP4-C2 from wheat and SP5-C1, SP5-C2 from rice.

Antibiotic susceptibility testing revealed that all isolates were susceptible to kanamycin and azithromycin. Eight isolates resistant to ampicillin and two isolates were susceptible (Fig 10). The detailed susceptibility pattern is presented in Table 2. Resistance to ampicillin may be associated with inherent β-lactamase production or adaptive resistance mechanisms, a frequent occurrence in persistent bacterial contaminants of in vitro cultures (Fang & Hsu, 2012). The observed sensitivity to kanamycin and azithromycin suggests potential use of these antibiotics to manage bacterial contamination in tissue culture systems (Tewelde *et al.,* 2020).

According to a another study, utilizing antibiotics to suppress and proliferate gram-negative bacteria was either difficult or ineffective (Zeinab *et al.,* 2023). The gram-negative bacteria in this study were sensitive to several antibiotics, which is a promising sign to limit the contamination of gram-negative bacteria during plant in vitro culture. Therefore, the sensitivity test and culture may be useful in determining how to regulate these organisms in tissue culture. However, long-lived use of antibiotic might have harmful side effects on explants and slow down the plant growth. (El-Banna *et al.,* 2021). Although careful optimization is necessary to avoid phytotoxic effects (Costa *et al.,* 2024). Based on systematic characterization of contaminants these findings strengthen the importance of antibiotic selection to improve micropropagation success.

Table 1: Cultural, morphological and biochemical characteristics of selected isolates

Isolates	Colony Shape	Colony Color	Morphology	Gram reaction	Biochemical Tests						Tentatively Identified Bacteria
					Catalase	Oxidase	Urease	Indole Production	Gelatin Hydrolysis	H_2S Production	
SP1-C1	Circular	White creamy	Rod	+	+	_	_	_	+	_	*Bacillus* spp.
SP1-C2	Circular	White creamy	Rod	+	+	_	_	_	+	_	*Bacillus* spp.
SP2-C1	Circular	Golden yellow	Coccus	+	+	_	+	_	+	_	*Staphylococcus* spp.
SP2-C2	Circular	Golden yellow	Coccus	+	+	_	+	_	+	_	*Staphylococcus* spp.
SP3-C1	Circular	White creamy	Rod	+	+	_	_	_	+	_	*Bacillus* spp.
SP3-C2	Circular	White creamy	Rod	+	+	_	_	_	+	_	*Bacillus* spp.
SP4-C1	Circular	White creamy	Rod	-	+	+	_	_	+	_	*Pseudomonas* spp.
SP4-C2	Circular	Golden yellow	Coccus	+	+	_	+	_	+	_	*Staphylococcus* spp.
SP5-C1	Circular	White creamy	Rod	-	+	+	_	_	+	_	*Pseudomonas* spp.
SP5-C2	Circular	Golden yellow	Coccus	+	+	_	+	_	+	_	*Staphylococcus* spp.

Note: (+) = positive result, (-) = Negative result, SP1-C1, SP1-C2 from aloe vera, SP2-C1, SP2-C2 from orchid, SP3-C1, SP3-C2 from banana, SP4-C1, SP4-C2 from wheat and SP5-C1, SP5-C2 from rice.

Table 2: Susceptibility pattern of isolated bacteria against three antibiotic discs

Isolates	K (30µg)			Azm (30µg)			Amp (25µg)		
	SDS	ZI	Result	SDS	ZI	Result	SDS	ZI	Result
SP1-C1	18 or more	29	S	18 or more	25	S	26 or more	10	R
SP1-C2	18 or more	29	S	18 or more	30	S	26 or more	0	R
SP2-C1	18 or more	30	S	18 or more	30	S	26 or more	0	R
SP2-C2	18 or more	25	S	18 or more	20	S	26 or more	20	R
SP3-C1	18 or more	29	S	18 or more	26	S	26 or more	0	R
SP3-C2	18 or more	27	S	18 or more	25	S	26 or more	0	R
SP4-C1	18 or more	27	S	18 or more	22	S	26 or more	20	R
SP4-C2	18 or more	29	S	18 or more	22	S	26 or more	21	S
SP5-C1	18 or more	31	S	18 or more	25	S	26 or more	20	S
SP5-C2	18 or more	30	S	18 or more	26	S	26 or more	0	R

Note: K- Kanamycin, Amp- Ampicillin, Azm- Azithromycin, SDS- Standard Diameter for susceptibility in millimeter, ZI- Zone of inhibition, S- Susceptible, R- Resistant, SP1-C1, SP1-C2, SP3-C1, SP3-C2= *Bacillus* spp., SP2-C1, SP2-C2, SP4-C2, SP5-C2= *Staphylococcus* spp., SP4-C1, SP5-C1= *Pseudomonas* spp.

In contaminated plant tissue cultures this study demonstrates the presence of a diverse bacterial population, encompassing gram-positive *Staphylococcus* and *Bacillus* species and gram-negative *Pseudomonas* species. The advancement of rod-shaped Gram-positive isolates aligns with previous observations indicating *Bacillus* as a common epiphyte and endophyte in-vitro plant systems (Romadanova *et al.,* 2022). Different biochemical responses underscore the needed for systematic identification to design targeted control strategies of contamination (Masi *et al.,* 2021). In plant tissue culture bacteria resistance to ampicillin observed among several isolates highlights a research gap in understanding adaptive resistance mechanisms, it indicates the need for further studies focusing on the molecular characterization and alternative way approaches. Implementation of these strategies could be significantly enhancing the efficiency and reliability of micropropagation protocols.

Conclusion

The current investigation demonstrated the presence of a wide range of contaminated bacteria in plant tissue culture systems, primarily associated with the genera *Bacillus, Staphylococcus, and Pseudomonas*. These isolates showed variable biochemical and morphological traits, indicating the heterogeneous

nature of in vitro microbial contaminant. Antibiogram profiling indicated that all isolates were sensitive to azithromycin and kanamycin but two were sensitive, eight were resistant to ampicillin. Based on specific sensitivity patterns it suggests the importance of antibiotic selection. The identification of both gram-negative and gram-positive bacteria underscore the necessity of stringent aseptic practices and continuous monitoring to minimize risks of contamination. Overall, these findings contribute to a better understanding of bacterial contamination in tissue culture and provide a direction for developing optimized decontamination strategies to enhance the reliability and success of plant micropropagation.

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