Identification and elimination of bacterial contamination during *in vitro* propagation of *Guadua angustifolia* Kunth

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ABSTRACT

Background: *Guadua angustifolia* Kunth is a very important bamboo species with significant utility in pharmaceutical, paper, charcoal, and construction industries. Microbial contamination is a major problem encountered during establishment of *in vitro* cultures of *Guadua*. **Objective:** This study has been designed to analyze the identity of contaminating bacteria and to develop the strategy to eliminate them during micropropagation of *Guadua*. **Materials and Methods:** We isolated and consequently analyzed partial sequence analysis of the 16S rRNA gene to identify two contaminating bacteria as (1) *Pantoea agglomerans* and (2) *Pantoea ananatis.* In addition, we also performed antibiotic sensitivity testing on these bacterial isolates. **Results:** We identified kanamycin and streptomycin sulfate as potentially useful antibiotics in eliminating the contaminating bacteria. We grew shoots on multiplication medium containing BAP (2 mg/l) and adenine sulfate (10 mg/l) supplemented with kanamycin (10 μg/ml) for 10 days and transferred them to fresh medium without antibiotics and found that bacterial growth was inhibited. Moreover, we observed intensive formation of high-quality shoots. Streptomycin sulfate also inhibited bacterial growth but at higher concentration. We also demonstrated that shoots grown in streptomycin sulfate tended to be shorter and had yellow leaves. **Conclusion:** Thus, we have developed a novel strategy to identify and inhibit intriguing microbial contaminations of (1) *Pantoea agglomerans* and (2) *Pantoea ananatis* during establishment of *in vitro* cultures of *Guadua*. This would improve *in vitro* establishment of an important bamboo, *Guadua angustifolia* Kunth for large scale propagation.

Key words: 16S rRNA gene sequencing, bacterial contamination, *Guadua angustifolia* Kunth, *in vitro* propagation

INTRODUCTION

Guadua angustifolia Kunth is one of the three largest and most important bamboo species in the world. Bamboo tar oil, recovered as a secondary product during the carbonization process, has significant medicinal value due to its antibiotic and antioxidant activities. *Guadua* also have a great potential to fix atmospheric carbon dioxide. Due to its versatility, lightness, flexibility, endurance, hardness, strength, climatic adaptability, seismic-resistance, rapid growth, and easy handling, it is widely employed in pharmaceutical, paper, charcoal, and construction industries.

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Bamboo culms are traditionally harvested from natural forests, but overexploitation has led to rapid depletion of their natural vegetative strands. As a result, most of the area under tropical rain forests and biodiversity has vanished and millions of hectares have been transformed into pastures and croplands. Therefore, there is a great concern about the conservation of bamboo's natural populations and thus need to develop novel propagation methodologies for new plantations and re-establishment of cleared strands.[1] The traditional propagation method by "offsets" limits the number of propagules. Moreover, the use of nodal segments for propagation is cumbersome and labor intensive for large-scale establishment of bamboo plantations.[2] Due to profound difficulties in the conventional propagation of bamboos, it is imperative to adapt alternative methods for rapid multiplication, and therefore micropropagation offers a feasible alternative.

Our attempts to obtain aseptic *in vitro* cultures using explant of *Guadua* from glasshouse-grown plants and to optimize a micropropagation procedure were hindered by persistent appearance of bacterial contamination in the cultures. Bacterial contamination in tissue culture is well documented,[3] and the failure of surface sterilization procedures to produce aseptic cultures is a major problem with woody plants.[4] Different experimental procedures including chemical sterilization and antibiotics have been used at various levels of success to minimize or eliminate such contamination. However, the type, concentration, and duration of antibiotic treatment vary for different plant tissue cultures. Therefore, it is pertinent to optimize antibiotic treatment strategy before its use.[5,6]

The growth medium selected for *in vitro* propagation also serves as a good source of nutrients for microbial growth. These microbes further compete adversely with plants for nutrients.[7] The presence of microbes or latent infections in these plant cultures usually results in increased culture mortality, variable growth, tissue necrosis, reduced shoot proliferation, and reduced rooting.[8]

We observed bacterial contamination in micropropagation of *Guadua angustifolia*. The contaminants were evident at the culture establishment stage and resulted in the loss of plants when bacteria overgrew the explants. In the present study, we have characterized two bacteria from *Guadua* shoot cultures, and determined effects of various antibiotics on these bacteria without adversely affecting the health of *in vitro* grown plant material.

MATERIALS AND METHODS

Disinfestation procedure

We used nodal segments measuring 2-4 cm cm in length from 4-year-old potted plants for initiating aseptic cultures. Briefly, we subjected explants to repeated washings after removal of leaf sheaths. This would remove all the adhering dust particles and microbes from the surface. The explants were then cleaned with a liquid detergent (Tween 20-HIMEDIA, Mumbai, India) followed by treatment with a suitable fungicide (e.g., Bavistin, 0.2%). Under sterile conditions in a laminar air flow bench, these explants were sterilized with 70% ethanol (v/v) and soaked in 0.04% $HgCl_2$.

Initiation of aseptic cultures

The sterilized explants were placed vertically in test tubes containing the MS (Murashige and Skoog's) medium[9] supplemented with BAP (6-Benzylaminopurine, 2mg/l), sucrose (2%), and agar (Murashige and Skoog 1962). The pH was adjusted to 5.7 prior to autoclaving. The cultures were incubated at a photosynthetic photon flux density (PPFD) of 70 ± 5 μmol/m^2/s from cool, white, fluorescent lamps at 25 ± 2°C. Moreover, the day length was maintained at 16 hours in a 24-hour light/dark cycle.

Isolation and identification of bacteria

Bacterial growth appeared as a cloudy zone in the agar medium around the shoot base within 20 days invariably in all the cultures. The contaminating bacteria were isolated by placing material with loop from visibly contaminated culture directly on the LB medium. After incubation at 28°C for 24 hours, two types of colonies were observed. Pure cultures of these bacteria were obtained by picking up the colonies and streaking them onto the fresh medium. These cultures were further maintained in glycerol stock at −80°C.

Antibiotic treatment of plants

The luria agar plates containing different antibiotics like kanamycin, carbenicillin, ampicillin, rifampicin, and streptomycin sulfate were inoculated with isolated bacterial contaminants for antibiotic sensitivity screening.

We selected two antibiotics, kanamycin, and streptomycin sulfate, on the basis of their effectiveness in antibiotic sensitivity testing. To test their effectiveness in eliminating bacterial contamination, the antibiotics were added to the multiplication medium, i.e., the liquid MS medium containing BAP (2 mg/l) and adenine sulfate (10 mg/l) in the following dosages: 0, 5, 10, 15, 25, 40, 50 g/ml alone and in combinations. Contaminated explants were then dipped in this medium for 10 days. Controls (plant tissue grown in the multiplication medium without antibiotics) were also included with each experiment. After 10 days of the antibiotic treatment, the physical conditions of plants were noted again and then placed in the liquid multiplication medium without any antibiotic. Shoots with no detectable signs of bacterial contamination were individually transferred onto the fresh medium without antibiotics and subcultured every 3 weeks. Growth rate and plant appearance were monitored to determine whether the antibiotics had any phytotoxic effects on plants during the multiplication and rooting phase.

16S rRNA gene sequencing

We isolated bacterial DNA from pure culture and performed PCR amplification of almost the entire length of 16S rRNA gene fragment. We used following primers 5'-AGAGCTTTGATCATGGCTCAGA-3' and 5'-GTTACCTTGTTACGACTT-3' to amplify 8 to28 and 1493 to 1510 parts of 16S rRNA gene of *Escherichia coli* and are useful for amplifying the 16S rRNA gene from various kinds of bacteria. The PCR was performed and analyzed on an agarose gel as described earlier.[10]

The 16S rRNA gene of bacteria was further sequenced to analyze its identity. Briefly, the amplified 16S rRNA gene was purified from the agarose gel using a Nucleospin Extract II kit. The PCR-purified product was directly used for nucleotide sequencing of the gene by using a Big Dye^R Terminator Cycle sequencing kit (Applied Biosystems). To identify bacteria, preliminary searches in the NCBI database were performed with BLASTIN program (http://www.ncbi.nlm.nih.gov/BLAST/, NCBI, Bethesda, MD, USA).

RESULTS AND DISCUSSION

The bacterial contamination encountered during *in vitro* propagation of plants is a major bottleneck which obstructs successful experimentation and establishment of aseptic cultures. Serious attempts have been made to develop procedures for eliminating these bacterial contaminants through (1) rigorous manipulation of environmental and nutritional factors or (2) treatment with antibiotics. [11] The association of bacteria with *in vitro* cultures of different crop plants, like watermelon, grape, banana, papaya, and capsicum has been encountered. This has been the cause of decline in the performance of cultures, degeneration of long-term maintained stocks, and lack of reproducibility of tissue culture protocols.[12-15]

We utilized 16S rRNA gene sequence analysis to identify bacterial contaminants [Figure 1] in *Guadua angustifolia* Kunth. These contaminants were found to be highly similar to *Pantoea agglomerans* (NCBI # FR872702) and *Pantoea ananatis* (NCBI # FR872704). Both bacteria are gram negative and closely related.[16,17] *P. ananatis* is a common epiphyte. It infects both monocotyledonous and dicotyledonous plants. It also occurs endophytically in hosts where it has been reported to cause disease symptoms.

Figure 1: Bacterial contamination in the region around shoot base in the agar medium.The bacteria appeared as creamish white growth around the base of shoots in the agar gelled medium after 20 days of inoculation

Apart from being associated with plants as an epiphyte, pathogen, or symbiont, it also occupies diverse and unusual ecological niches where it may function as a saprophyte. *P. agglomerans* is known to be an opportunistic pathogen in the immunocompromised, causing wound, blood, and urinary tract infections. It is commonly isolated from plant surfaces, seeds, fruits (namely mandarin oranges), and animal or human feces.

Many bacteria grow slowly or not at all in media used in plant culture, thus escaping detection until considerable time and materials have been invested.[18] The ideal approach is to use antibacterial substances (e.g., antibiotics) but it has met with varying degrees of success.[3,19] In many cases, antibiotics have been found to be phytotoxic at high concentrations enough to destroy all contaminants.[3,20] The lack of descriptive information and antibiotic susceptibilities of a large number of plant-associated bacteria further complicate the use of antibiotics.[21] Therefore, the characterization and identification of plant-associated bacteria can lead to more successful antibacterial therapies.[19]

Our antibiotic sensitivity testing revealed kanamycin and streptomycin sulfate as the most effective antibiotic against the contaminating bacteria [Table 1]. The kanamycin was least phytotoxic during micropropogation of *G. angustifolia*. The shoot tips were grown for 10 days on the multiplication medium containing the kanamycin (10 μg/ml). The addition of kanamycin grossly inhibited the bacterial growth while allowing the formation of high-quality *Guadua* shoots [Figure 2]. In contrast, streptomycin was effective at reducing bacterial growth in tissue culture at higher concentrations (15 μg/ml). Moreover, the shoot number and the quality of *Guadua* were also reduced. Such inhibition of shoot growth by streptomycin has also been noted during micropropagation of *Pelargonium*.[22]

Kanamycin interacts with the 30S subunit of prokaryotic ribosomes. It induces substantial amount of mistranslation and indirectly inhibits translocation during protein synthesis. Streptomycin binds to the S12 protein of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. This prevents initiation of protein synthesis and leads to death of microbial cells. It may also inhibit protein synthesis in chloroplasts and mitochondria in plant tissues, and thus resulting in small and yellow leaves.

Traditionally, combinations of antibiotics have been used against bacterial contaminants in plant tissue culture.[3,23] The combinations of two or more antibiotics for eliminating bacterial contaminants are very well recommended.[4,22] However, we interestingly found kanamycin (10 μg/ml)

Table 1: The effect of various antibiotics on the growth of bacteria and *Guadua angustifolia* Kunth

Antibiotic	Concentration (µg/ml)	Removal of bacteria	Health status of shoots
Streptomycin sulfate	0	−	Overgrowth of bacteria inhibited shoot survival
	5	+	Overgrowth of bacteria inhibited shoot survival
	10	++	Shoots were fresh, green, and healthy
	15	+++	Shoots were yellowish green with small leaves
	25	+++	Shoot necrosis
	40	+++	Shoot necrosis
	50	+++	Shoot necrosis
Kanamycin	0	−	Over growth of bacteria inhibited shoot survival
	5	++	Overgrowth of bacteria inhibited shoot survival
	10	+++	Shoots were fresh, healthy, and green
	15	+++	Shoots were yellowish green
	25	+++	Shoot necrosis
	40	+++	Shoot necrosis
	50	+++	Shoot necrosis

"+" sign denotes a positive response to some extent; "++" sign denotes a positive response to moderate extent; "+++" sign denotes a complete positive response; "−" sign denotes no response

Figure 2: The growth of healthy *G. angustifolia* shoots in the multiplication medium after treatment with kanamycin. The treatment of shoots with kanamycin (10 µg/ml) grossly inhibited the bacterial growth without affecting their quality and shoot numbers

Figure 3: Acclimatized plants of *G. angustifolia.* Plantlets were transferred to plastic pots containing sand in polytunnels and covered with jars to maintain high relative humidity. After 1 month of hardening, these plants demonstrated 90% survival when transferred to pots containing 1:1:1 mixture of soil, sand and manure

as very effective in eliminating bacterial contaminants with least phytotoxicity. Such shoots with no detectable signs of bacterial contamination were transferred onto the fresh multiplication medium without antibiotic after every 3 weeks. The multiplication rate of shoots treated with antibiotic was similar to that of healthy plants. These shoots were able to produce healthy roots in the same multiplication medium without addition of auxin. These plants were successfully hardened under green house conditions [Figure 3].

CONCLUSION

The common problem of bacterial growth around *in vitro* shoots in *Guadua angustifolia* tissue culture is due to (1) *Pantoea agglomerans* and (2) *Pantoea ananatis*. This can be resolved through treatment of *G. angustifolia* shoots with kanamycin for 10 days. No phytotoxicity appeared when shoots were treated with kanamycin (10 µg/ml) and the multiplication rate of treated *G. angustifolia* shoots was found to be similar to that of healthy plants. Streptomycin sulfate, at higher concentration, also inhibited bacterial growth during micropropagation of *G. angustifolia*. In addition, shoots grown in streptomycin sulfate tended to be shorter and have stunted leaves. Thus, our study provides us with a technique to identify and resolve bacterial contamination of (1) *Pantoea agglomerans* and (2) *Pantoea ananatis* during *in vitro* culture of *G. angustifolia*.

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REFERENCES

1. Judziewicz EJ, Clark LG, Londono X, Stem M. American bamboos. Washington: Smithsonian Institution Press; 1999. p. 392.
2. Sood A, Ahuja PS, Sharma M, Sharma OP, Godbole S. *In vitro* protocols and field performance of elites of an important bamboo *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro. Plant Cell Tissue Organ Cult 2002;71:55-63.
3. Leifert C, Camotta H, Waites WM, Cheyne VA. Elimination of *Lactobacillus plantarum*, *Corynebacterium* spp., *Staphylococcus saprophyticus* and *Pseudomonas paucimobilis* from micropropagated Hemerocallis, Choisya and Delphinium cultures using antibiotics. J Appl Bacteriol 1991;71:307-30.
4. Reed BM, Mentzer J, Tanprasert P, Yu X. Internal bacterial contamination of micropropagated hazelnut: identification and antibiotic treatment. Plant Cell Tissue Organ Cult 1998;52:67-70
5. Phillips R, Arnott SM, Kaplan SE. Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explants cultures of *Helianthus tuberosus*. Plant Sci Lett 1981;21:235-240.
6. Buckley PM, DeWilde TN, Reed BM. Characterization and identification of bacteria isolated from micropropagated mint plants. *In Vitro* Cell Dev Biol Plant 1995;31:58-64.
7. Odutayo OI, Amusa NA, Okutade OO, Ogunsanwo YR. Determination of the sources of microbial contaminants of cultured plant tissues. Plant Pathol J 2007;6:77-81.
8. Kane M. Bacterial and fungal indexing of tissue cultures. Available from: http://www.hos.ufl.edu/moorweb/TissueCulture/class1/Bacterial%20and%20fungal%20indexing%20of%20tissue%20cultures.doc. [Last accessed on 2003].
9. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473-97.
10. Salwan R, Gulati A, Kasana RC. Phylogenetic diversity of alkaline protease-producing psychrotrophic bacteria from glacier and cold environments of Lahaul and Spiti, India. J Basic Microbiol 2010;50:150-9.
11. DeFossard RA, DeFossard H. Coping with microbial contaminants and other matters in a small commercial micropropagation laboratory. Acta Hortic 1988;225:167-76.
12. Thomas P. A three-step screening procedure for detection of covert and endophytic bacteria in plant tissue cultures. Curr Sci 2004;87:67-72.
13. Thomas P. *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long-term micropropagated triploid watermelon cultures. Plant Cell Tissue Organ Cult 2004;77:173-9.
14. Thomas P. Isolation of *Bacillus pumilus* from *in vitro* grapes as a long-term alcohol-surviving and rhizogenesis inducing covert endophyte. J Appl Microbiol 2004;97:114-23
15. Thomas P. Isolation of an ethanol-tolerant endospore-forming Gram-negative *Brevibacillus* sp. as a covert contaminant in grape tissue cultures. J Appl Microbiol 2006;101:764-74.
16. Hauben L, Moore ER, Vauterin L, Steenackers M, Mergaert J, Verdonck L, *et al*. Phylogenetic position of phytopathogens within the Enterobacteriaceae. Syst Appl Microbiol 1998;21: 384-97.
17. Mergaert J, Verdancy L, Keister K. Transfer of *Erwinia ananas* (synonym *Erwinia uredovora*) and *Erwinia stewartii* to the genus *Pantoea* emend as *Pantoea ananas* (Serrano 1928) comb. nov. and *Pantoea stewartii* (Smith 1898) comb. nov., respectively, and description of *Pantoea stewartii* subsp. *indologene*s subsp. nov. Int J Syst Bacteriol 1993;43:162-73.
18. Viss PR, Brooks EM, Driver JA. A simplified method for the control of bacterial contamination in woody plant tissue culture. *In Vitro* Cell Dev Biol Plant 1991;27:42.
19. Cornu D, Michel MF. Bacterial contamination in shoot cultures of *Prunus avium* L. Choice and phytotoxicity of antibiotics. Acta Hortic 1987;212:83-6.
20. Leifert C, Camotta H, Waites WM. Effect of combinations of antibiotics on micropropagated Clematis, Delphinium, Hosta, Iris and Photinia. Plant Cell Tissue Organ Cult 1992;29:153-60.
21. Leifert C, Waites WM, Nicholar JR. Bacterial contaminants of micropropagated plant tissue cultures. J Appl Bacteriol 1989;67:353-61.
22. Wojtania A, Pulawska J, Gabryszewska E. Identification and elimination of bacterial contaminants from Pelargonium tissue cultures. J Fruit Ornamental Plant Res 2005;13:101-8.
23. Young PM, Hutchins AS, Canfield ML. Use of antibiotics to control bacteria in shoot cultures of woody plants. Plant Sci Lett 1984;34:203-9.

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