

Identification Bacterial Contaminant in *Semiarundinaria fastuosa* Tissue Culture

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Abstract. Bamboo is one of the plants which propagated by tissue culture technique however, the emergence of microbial contaminant caused decrease of bamboo production. A type of microbe that caused the contamination on *Semiarundinaria fastuosa* tissue culture is bacteria. This study aimed to isolate bacterial contaminant and understanding its biodiversity. Bacterial isolation and phenotypic characterization were done by observing morphology, physiological test, biochemical test, identification and numeric phenetic analysis. Twelve bacterial contaminants was isolated and based on profile matching with *Bergey's Manual of Determinative Bacteriology*, six isolate was a member of genus *Bacillus*, one isolate *Enterococcus*, two isolate *Xenorhabdus*, one isolate *Morococcus*, one isolate *Corynebacterium*, and one isolate belongs to genus *Sarcina*. A dendrogram created using *Simple Matching coefficient* (Ssm) parameter and average linkage algorithm shown that on the 71% similarity index, all 12 OTU was grouped in one cluster. However, a dendrogram produced by *Jaccard's coefficient* (Si) parameter and average linkage algorithm on 70% similarity index divided 12 OTU on to 12 clusters.

Keywords: *Semiarundinaria fastuosa* tissue culture, bacterial contaminants, numerical-fenetic classification

INTRODUCTION

Plant tissue culture is a technique of growing cells, tissues, and plant organs in liquid and solid media that contain nutrients in aseptic conditions (Devi & Srinivasan, 2006). Propagation of plants by tissue culture techniques (in vitro) will produce plant seeds in large quantities in a relatively short time so it is more economical. The success of this technique is influenced by a variety of factors, including: micro and macro nutrients, explant sources, administration of growth regulators (PGR), condition of materials, equipment, and sterile spaces. In the life of Indonesian people, bamboo is one of the natural resources which has a very important role. Bamboo has many uses, including ornamental plants, house fences, erosion prevention, household crafts, and foodstuffs (Coal, 2002). Bamboo can be propagated by plant tissue culture methods. One of the problems faced in bamboo tissue culture is contamination.

Bacteria are a type of microbes that usually become contaminants in plant tissue culture (Barbara & Piyaarak, 1995). Sources of bacterial contaminants include lack of aseptic techniques; inadequate sterilization of tools, media and explants; working room; operator; and from explants (the presence of endophytic and epiphytic bacteria) (Buckley et al., 1995). Therefore, in this study characterization and identification of contaminant bacteria will be carried out in bamboo tissue culture types of *Semiarundinaria fastuosa* in order to know the types of contaminant bacteria. The benefits of knowing

the types of contaminant bacteria so that it can be known ways to prevent the entry of contaminant bacteria in bamboo tissue culture so that bacterial contaminants in bamboo tissue culture can be minimized.

MATERIALS AND METHODS

Materials

Ringer's solution, nutrient agar, nutrient broth, gram staining, spore staining, SIM medium, H₂O₂ 3%, Simmon's citrate medium, Starch agar, nutrient gelatine, MR-VP medium, nitrate reduction medium, Gula-gula medium, NaCl, H₂SO₄, HCl.

Methods

1. Samples from solid media: bacterial colonies that are seen are taken using loop, then diluted with Ringer's solution to 10⁻⁸ dilutions. While for liquid samples 1 ml is taken, diluted with Ringer's solution to 10⁻⁸ dilutions. Samples planted for bacterial isolation from dilutions of 10⁻¹ to 10⁻⁸ each of 2 replications.
2. Isolate contaminant bacteria with Nutrient Agar Plate medium using the spread plate method
3. Purification of bacteria using the streak plate method to obtain pure culture
4. Characterization of bacterial isolates include: Observation of cell morphology for all isolates using the Hans Christian Gram gram staining method. For gram-positive isolates continued with spore painting using the Schaeffer-Fulton method

5. Observation of the morphology of the colonies of all isolates by planting isolates on a medium agar plate, stab agar, and slant agar
6. Test the oxygen requirements of the isolate by planting the isolate on a nutrient broth medium
7. Test catalase, motility, H₂S production, and indole with SIM (Sulfur Indole Motility) medium
8. Simmon's Citrate Test, starch hydrolysis, gelatin hydrolysis, urea hydrolysis, MR-VP (Methyl Red and Voges-Proskauer), nitrate reduction, and carbohydrate fermentation including glucose, sucrose, lactose, galactose, fructose, maltose and mannitol
9. Test the effect of temperature, salinity, and pH on the growth of bacterial isolates
10. Identification of bacterial isolates by matching profile methods with the biochemical properties of each reference genus according to Bergey's Manual of Determinative Bacteriology

RESULTS AND DISCUSSION

Bacterial Isolate Phenotype Character

In this study, 12 contaminant bacterial isolates were obtained in *Semiarundinaria fastuosa* tissue culture. Based on the table of phenotypic characters, seen from the cell shape, there are 3 isolates in the form of coccus and 9 isolates in the form of rods. 9 isolates included in the group of gram-positive bacteria while 3 isolates included in the group of gram-negative bacteria. Most of the isolates including gram-positive bacteria because gram-positive bacteria may have spores so that they are more resistant to various environments. Furthermore, all gram-positive bacterial isolates were painted spores to find out whether the bacteria had spores or not. Results of spore painting showed that 6 bacterial isolates had spores while 6 isolates did not have spores. Bacteria that have spores can live in a variety of environmental conditions, therefore contaminant bacteria are mostly bacteria that have spores.

Based on oxygen requirements, as many as 5 isolates including aerobic bacteria, 1 isolate including anaerobic bacteria, 6 isolates of bacteria including facultative anaerobic bacteria. Most contaminant bacteria include facultative anaerobic bacteria because this group of bacteria can adjust to the oxygen levels available in their environment. In the test of the ability to use citrate as a carbon source, none showed positive results. Motility test results of 11 isolates including motile bacteria and 1 isolate including non-motile bacteria. The catalase test was 4 negative isolates, while 8 isolates were positive. This shows that the isolate has a catalase enzyme that can degrade hydrogen peroxide into oxygen and water with the following reaction: $2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2$

Based on the test of the effect of temperature on bacterial growth, no isolates were able to grow at all temperatures tested, ie from -4 °C, 4-5 °C, 25 °C, 37 °C, and 75 °C. Isolates that grow at temperatures of -4 °C, 4-5 °C, 25 °C, 37 °C, and 75 °C respectively, 1, 2, 12, 12, and 7 isolates. All isolates included mesophyll bacteria because they grew optimally at temperatures between 20 °C to 50 °C. Most pathogenic bacteria grow optimally at temperatures between 35 °C to 40 °C. In the nitrate reduction test, 4 isolates showed positive reactions and 8 isolates showed negative reactions. Some facultative anaerobic bacteria can use nitrate as the last electron acceptor in anaerobic respiration called nitrate respiration. The enzyme involved in nitrate reduction is the nitrate reductase enzyme. The chemical reaction for reducing nitrates to nitrites is as follows:



In the test of the effect of pH all isolates can grow at pH 7. Bacteria that can grow at pH 7 or pH close to 7 are called neutrophiles. In the test of the effect of salinity on growth, bacteria that are able to survive on the salinity of 0.5%, 1%, 3%, 5%, 7%, 9%, 11%, 13%, and 15% respectively 12, 12, 10, 9, 4 and 3 isolates. The higher the salt content the less the number of bacteria that is able to grow.

Diversity of Bacterial Isolates in Bamboo Tissue Culture Contaminants

After all isolates were characterized, identification was then performed. Identification of contaminant bacterial isolates in *Semiarundinaria fastuosa* culture was carried out using the profile matching method by referring to the key characters in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) to determine which bacterial contaminants in *Semiarundinaria fastuosa* culture belong to what genus. The profile matching results showed that 12 isolates were divided into 6 genera, including: *Bacillus*, *Enterococcus*, *Xenorhabdus*, *Sarcina*, *Morococcus*, and *Corynebacterium*. The genus *Corynebacterium* is a type of bacterial contaminant in plant tissue culture (Bastiaens, 1983), *Bacillus* (Bastiaens, 1983; Kotiranta et al., 2000; Coenye & Vandamme, 2003; Johansen et al., 2005; Devi, 2006; Janssen, 1983; 2006; Compant et al., 2008).

The results of profile matching of all bacterial contaminants of bamboo culture contaminants were 50% (6 isolates) belonging to the genus *Bacillus* spp. Isolates included in the genus *Bacillus* have key characters: gram positive (+), rod shape, berspora, motile, positive catalase, aerobic / facultative anaerobes. The habitat of the genus *Bacillus* includes soil, water, food, marine sediments, manure, acidic foods, and lake sediments (Slepecky & Hemphill, 2006). *Bacillus* spp becomes one of the dominant bacteria as contaminants in various plant tissue cultures, possibly because *Bacillus* spp cells

produce endospores that can withstand temperatures of 100 ° C or more and are very resistant to disinfectants such as alcohol, hypochlorite, and mercury chloride (Claus & Berkeley 1986, Kunnemann & Faaji-Groenen, 1988).

Isolates included in the genus *Enterococcus* spp were 8.33% (1 isolate) with key characters: coccus / round, non-sporical, gram positive, motile, facultative anaerobic, negative catalase, and usually grew optimally at 37° C. Habitat *Enterococcus* spp is in clinical waste, commonly found in animals and humans because it is a microflora in the human body and animals (Fisher & Phillips, 2009). Apart from being a bacterial contaminant in bamboo culture, *Enterococcus* spp is also known as a bacterial contaminant in several types of plant tissue cultures of the genus *Coffea*, *Iris*, *Hevea*, *Choisya*, *Pulmonaria*, and *Hemerocallis* (Leifert et al., 1994).

A total of 8.33% (1 isolate) belongs to the genus *Corynebacterium* sp with key characters: gram positive, some are motile or non-motile, do not form endospores, aerobes, and positive catalase (Holt et al., 1994). This group of bacteria is pathogenic to plants, which causes various diseases by showing signs of wilting plants (Davis, 1986; Stange, 1996; Vidaver, 1979). The isolates included in the genus *Xenorhabdus* spp were 2 isolates (16.6%) with key characters: gram negative, non-sporical, rod shaped, facultative anaerobic, motile, negative catalase (Holt et al., 1994). *Xenorhabdus* spp turns out to form a symbiosis with entomopathogenic nematodes namely *Steinema* (Boemare, 2002). This is a form of bacterial symbiosis that contributes to the pathogenicity of nematode hosts (Herbert & Goodrich-Blair, 2007).

One isolate (8.33%) included in the genus *Morococcus* sp had key characters: gram positive, coccus shaped, non-spore forming, non-motile, aerobic, and positive catalase. The isolates included in the genus *Sarcina* sp were only 1 isolate (8.33%) with key characters: gram positive, coccus shaped, non-spore forming, non-motile, anaerobic, and negative catalase. The genus *Sporolactobacillus* sp, *Sarcina* sp, *Listeria* spp, *Xenorhabdus* spp, *Trichococcus* spp, and *Morococcus* sp are not yet known whether they have been found as contaminant bacteria in tissue culture of plants other than bamboo.

CONCLUSIONS

In this research successfully carried out isolation of bamboo tissue culture contaminants by obtaining 12 bacterial isolates. The results of profile matching showed that various types of bacterial contaminants from bamboo culture contaminants were obtained, namely the group of gram-positive bacteria including: *Bacillus*, *Corynebacterium*, *Enterococcus*, *Morococcus*, and *Sarcina*. The group of gram-negative bacteria is *Xenorhabdus*.

REFERENCES

- Barbara, M. Reed and Piyaarak Tanprasert. 1995. Detection and Control of Bacterial Contaminants of Plant Tissue Cultures. A Review of Recent Literature Ermayanti, T.M. 1997. Mengenal dan Mengatasi Kontaminan Pada Biak Jaring Tanaman. Warta Biotek Tahun XI No. 3, September-Desember 1997. Plant Tissue Culture and Biotechnology 137-142. Volume No.3. USA.
- Bastiaens, L. 1983. Endogenous Bacteria In Plants and Their implications In Tissue Culture- A Review. Med. Fac. Landbouww. Rijksuniv. Gent 48: 1-11.
- Boemare, N. 2002. Systematics of *Photorhabdus* and *Xenorhabdus* In R.Gaugler (Ed.), Entomopathogenic Nematology (pp. 35-56). CABI Publishing. New Brunswick.
- Buckley, P.M., DeWilde, T.N., and Reed, B.M. 1995. Characterization and Identification of Bacteria Isolated from Micropropagated Mint Plants. In vitro Cell. Dev. Biol. 31P: 58-64.
- Claus, D., and Berkeley, R. C. W. 1986. Description of The Genus *Bacillus* In: *Bergey's Manual of Systematic Bacteriology*. Pp. 1105-1139. Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. Eds. Williams & Wilkins. London, U.K.
- Coenye, T. and Vandamme, P. 2003. Diversity and Significance of *Burkholderia species* Occupying Diverse Ecological Niches. Environ. Microbiol. 5(9): 719-729.
- Compant, S., Nowak, J., Coenye, T., Clement, C., and Ait Barka, E. 2008. Diversity and Occurrence of *Burkholderia* spp. In The Natural Environment. Federation of European Microbiological Societies (FEMS). Microbiology Reviews 32(4) : 607-26.
- Davis, M. J. 1986. Taxonomy of Plant Pathogenic Coryneform Bacteria. Ann. Rev. Phytopathol. 24:115-140.
- Devi, C.S. and Srinivasan, V.M. 2006. Studies on Various Atmospheric Microorganisms Affecting the Plant Tissue Culture Explants. Academic Journals Inc., USA. American Journal of Plant Physiology I (2): 205-209.
- Fisher, Katie and Phillips, Carol. 2009. The ecology, epidemiology and virulence of *Enterococcus*. University of Northampton, School of Health, Park Campus, UK. Microbiology, 155, 1749-1757.
- Herbert, E. E., and Goodrich-Blair, H. 2007. Friend and Foe: The Two Faces of *Xenorhabdus nematophila*. Nat Rev Microbiol, 5 (8), 634-646.
- Holt, G.J., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T. 1994. *Bergey's Manual of Determinative Bacteriology* 9th Edition. USA: Williams and Wilkins Baltimore.
- Janssen, P.H. 2006. Identifying The Dominant Soil Bacterial Taxa In Libraries of 16S rRNA and 16S rRNA Genes. Applied and Environmental Microbiology 72(3) : 1,719-28.
- Johansen, J.E., Binnerup, S.J., Kroer, N., and Molbak, L. 2005. *Luteibacter rhizovicinus* gen. nov., sp. Nov., a yellow-pigmented gammaproteobacterium isolated from the rhizosphere of barley (*Hordeum vulgare* L.). International Journal of Systematic and Evolutionary Microbiology 55(6): 2, 285-291.
- Kotiranta, A., Lounatmaa, K., and Haapasalo, m. 2000. *Epidemiology and Pathogenesis of Bacillus cereus* Infections. Microbes and Infection 2(2):189-8.
- Kunnemann, B. P. A. M., and Faaij-Groenen, G. P. M. 1988. Elimination of bacterial contaminants: a matter of detection and transplanting procedures. Acta Hort. 225: 183-189.

- Leifert, C., Morris, C.E. and Waites, W.M. 1994. Ecology Of Microbial Saprophytes and Pathogens In Tissue Culture and Field-Grown Plants: Reasons For Contamination Problems In Vitro. *Critical Reviews in Plant Science* 13: 139-183.
- Slepecky, R. A. and Hemphill, H.E. 2006. The Genus *Bacillus*-Nonmedical. Chapter 1.2.16. *Prokaryotes* (2006) 4:530-562. DOI: 10.1007/0-387-30744-3_16.
- Stange, R. R. Jr., Jeffares, D., Young, C., Scott, D. B., Eason, J.R., and Jameson, P. E. 1996. PCR Amplification of The Fast-1 Gene for The Detection of Virulent Strains of *Rhodococcus fascians*. *Plant Path.* 45: 407-417.
- Vidaver, A. K., and Starr, M. P. 1979. Phytopathogenic Coryneform and Related Bacteria. Pages 1879-1887. In: M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel, (eds.). *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*. Springer-verlag, Berlin, Heidelberg, New York.