

First Report on Infection of *Eucalyptus pellita* Seeds by *Ralstonia solanacearum*[†]

Bayo A. Siregar^{1,2}, Abdul Gafur^{2*}, Pranita Nuri², H. Halimah², Budi Tjahjono² and G.D. Golani³

¹ Department of Plant Protection, IPB University, Jl. Kamper Wing 7 Level 5, Dramaga, Bogor 16680, Indonesia; bayo.alhusaeri@yahoo.com

² Sinarmas Forestry Corporate Research and Development, Perawang 28772, Indonesia

³ Sinarmas Forestry Technical Adviser, Jakarta, Indonesia

* Correspondence: gafur@uwalumni.com; Tel.: +62-761-900-0200

† Presented at the 1st International Electronic Conference on Forests, 15–30 November 2020;

Available online: <https://sciforum.net/conference/IECF2020>

Published: 25 October 2020

Abstract: Bacterial wilt is one of major threats to eucalyptus plantations which may cause significant losses. Until now, study about bacterial wilt on *Eucalyptus pellita* in Indonesia is still very limited, especially about the presence of the pathogen on or in the seeds. This study aims to provide evidence of the existence of the *R. solanacearum* bacterium on or in *E. pellita* seeds. Detection of seed-borne bacteria is determined by several approaches such as (i) direct detection using universal and selective medium in the laboratory, (ii) nursery test, and (iii) species-specific molecular detection. Results of our study indicate that *R. solanacearum* can be detected from eucalyptus seeds using universal and selective medium in the laboratory, nursery test, and molecular-based detection using the Enrichment PCR method. The bacterial inoculum is also proven to exist both on the surface of and inside the eucalyptus seeds. This is the first report that *R. solanacearum* is a seed-borne pathogen in *E. pellita* seeds. Previous studies in different agricultural systems show that the effective method used to control the pathogen is through seed treatments using biological, physical, and chemical approaches.

Keywords: bacterial wilt disease; detection; pathogen; plantation; seedborne.

1. Introduction

Forest plantation in Indonesia has been rapidly increasing since the last few decades and reached 11 million hectares last year [1]. Ecologically, forest plantations are able to reduce pressure on natural forests, quickly fix carbon and stimulate the restoration of natural vegetation. However, monoculture plantations are challenged to sustainably manage pest and disease risks [2]. Traditionally, the use of healthy (pathogen-free) seeds is one approach that can be taken to reduce the risk of disease. So far, most of the reports on seed-borne pathogens on forest trees have dealt with fungi only. Information on seed transmission of other pathogens, especially bacteria, was very limited [3]. Take for example the bacterial wilt pathogen of eucalyptus, *Ralstonia solanacearum*. The pathogen has previously been reported to be a seed-borne pathogen in many agricultural plants including eggplant, tomato, chili, potato, and ginger [4–6]. However, it was yet to be proved as seed-transmitted in eucalyptus. Bacterial wilt is one of major threats in eucalyptus plantations which can cause significant losses [7]. Until now, study about bacterial wilt on *E. pellita* in Indonesia is still very limited, especially regarding the presence of the pathogen on or in the seeds. This study aims to provide evidence of the existence of the *R. solanacearum* bacterium on or in *E. pellita* seeds. This is important as in different pathosystems the pathogen can be effectively controlled through seed treatments.

2. Materials and Methods

Seed testing was performed in laboratory using universal and selective media. *E. pellita* seeds (1 g) from different seed lot numbers were crushed using pestle and mortar and the sap liquid (0.1 ml) was taken for plating into TZC (2,3,5-triphenyl tetrazolium chloride) for universal medium and modified TZC for selective medium [8]. The agar plate was incubated at 28 °C for 48-72 hours. A single colony of irregularly shaped, fluid and round pink bacterium is positively the colony of virulent *Ralstonia*.

Nursery test was performed through germinating seeds of four different *E. pellita* seed lot numbers. The seedlings were maintained in the nursery until 60 days old. The seedlings were symptomless. A total of 60 healthy-looking seedlings were collected for pathogen isolation using the procedure described elsewhere [9]. The stems were cut transversely and placed in a soaked container and sterilized with 5.25% NaOCl twice before being rinsed with sterile water. The stem pieces were then inserted into a test tube containing 10 ml of sterile distilled water and incubated in an incubator shaker for 30 min. The water stem immersion (0.1 ml) was taken and grown on TZC media and incubated at 28° C for 72 hours.

Molecular detection was carried out through Enrichment PCR (En-PCR) using universal *Ralstonia* primer. The seeds from seven seed lot number were divided into two groups, with or without surface sterilization. Surface sterilization was conducted using NaOCl (5.25%) before the seeds were soaked in sterilized water. The sample (0.5 g) was crushed using pestle and mortar and the sap liquid (1 ml) was taken and added 3 ml of TZC Enrichment medium and incubated for 24-48 hours. Genomic DNA was extracted using the Bacteria Genomic DNA Kit (Geneaid) according to the manufacturer's instructions. To confirm that all tested strains belong to the complex *R. solanacearum*, PCR amplification was performed using species-specific primers 759 (5'-GTCGCCGTCAACTCACTTTCC-3') and 760 (5'-GTCGCCGTCAGCAATGCGGAATCG-3') to amplify a 280 bp fragment [10]. Each reaction mixture (25 µL) contained 1x reaction buffer, 1 U Taq DNA polymerase (Green GoTaq), 25 mM MgCl₂, 2.5 mM dNTP mix, 10 µM of each primer and 20 ng of DNA template. Amplification was carried out in a 2720 Thermal Cycler (Applied Biosystem). The cycling conditions used were: denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94°C for 30s, primer binding at 55°C for 30s, elongation at 72°C for 1 min; and a final elongation step at 72 °C for 5 min. Water was included in every PCR assay as a negative control.

3. Results and Discussion

Bacterial wilt infection in eucalyptus seedlings is usually without apparent symptoms or known as latent infection [8]. The nursery test and laboratory detection need to be done to quantify the infection rate in seedlings. Table 1 shows the presence of the *Ralstonia* bacterium in symptomless seedlings with infection rate varying between 6 and 42% depending on the seed lot number. These findings indicate the potential of bacterial wilt disease to be transmitted through seeds. Planting seedlings with latent infection into the field may cause an outbreak of the disease [11]. Seed health testing is therefore necessary to obtain pathological quality assurance, that the seeds are clean and not contaminated, to minimize pathogen dispersal through seed transmission [12].

Table 1. Infection rate (%) of *Ralstonia solanacearum* in symptomless *Eucalyptus pellita* seedlings

Seed Lot Number	Infection rate (%)
12073	42.4
12074	6.7 – 36.7
12075	8.6 – 19.7
12076	8.3

The seed samples without surface sterilization treatment produced positive bands, implying that they had the *Ralstonia* bacterium. A positive result was determined from the position of the band that

matched the expected product length. The amplified 281-bp-DNA fragment of each sample was obtained. Two of the seven seed lot samples with surface-sterilization treatment were positive, an indication that the bacterial inoculum was present both on the surface of and inside the eucalyptus seeds (endosperm) (Table 2).

Table 2. Detection of *Ralstonia solanacearum* in *Eucalyptus pellita* seeds using En-PCR

Seed Lot Number	En-PCR Result ¹	
	Surface	Endosperm
EP15216AA5	+	+
EP15215AA5	-	-
EP15219AA5	+	-
EP15214AA5	+	-
EP15218AA5	+	+
EP15217AA5	-	-
EP15211AA5	+	-

¹ + : *Ralstonia solanacearum* was positively detected using En-PCR.

PCR assay amplifies the DNA of target organisms, targeting the species-specific sequences in their genome. In the present study, an efficient DNA isolation protocol and PCR-based detection of the bacterial wilt pathogen in soil and infected plant materials were carried out using primers of 759F/760R. The primers were originally developed for amplification of different *R. solanacearum* isolates [10]. Our results confirmed the success of using these specific primers to locate *R. solanacearum*. Although the disease can be managed using consortium of antagonistic bacteria [13], detection of *R. solanacearum* at low concentration in seeds and plant tissues, which may be undetected using conventional methods, is very critical. Studies elsewhere show that seed treatment is also effective in controlling the pathogen [14].

4. Conclusion

R. solanacearum can be detected from eucalyptus seeds using universal and selective media in the laboratory, nursery test, and molecular-based detection using the Enrichment-PCR method. The bacterial inoculum is present both on the surface of and inside the eucalyptus seeds. This is the first report that *R. solanacearum* is a seed-borne pathogen in *E. pellita* seeds.

Author Contributions: Conceptualization, B.A.S.; methodology, B.A.S. and A.G.; formal analysis, B.A.S.; investigation, B.A.S., P.N. and H.H.; writing—original draft preparation, B.A.S.; writing—review and editing, A.G.; supervision, B.T. and G.D.G.

Funding: This research received no external funding.

Acknowledgments: The authors thank Sinarmas Forestry Corporate Research and Development (SMF R&D) for providing laboratory materials and analysis as well as Genetic Deployment and Forest Improvement Divisions of the SMF R&D for providing *E. pellita* seeds.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. BPS-Statistics Indonesia; Statistics of Timber Culture Establishment 2018; BPS-Statistics Indonesia: Jakarta, Indonesia, 2019; pp. 12-16.
2. Liu C.L.C.; Kuchma O.; Krutovsky K.V. Mixed-species versus monocultures in plantation forestry: Development, benefits, ecosystem services and perspectives for the future. *Glob. Ecol. Conserv.* 2018, 15, e00419.
3. Sutherland J.R.; Diekmann M.; Berjak. Forest Tree Seed Health. IPGRI Technical Bulletin No. 6; International Plant Genetic Resources Institute: Rome, Italy, 2002; pp. 9-10.

4. Sumithra K.; Krishnappa M.; Vasanth T.K.; Shetty H.S.; Mortensen C.N.; Mathur S. Seed-borne nature of *Ralstonia solanacearum* in eggplant (*Solanum melongena* L.) cultivars in India. *Seed Sci. Technol.* **2000**, *28*, 291-299.
5. Van der Wolf J.M.; De Boer S.H. Bacterial pathogen of potato. In *Potato Biology and Biotechnology, Advances and Perspectives*; Vreugdenhil D., Bradshaw J., Gebhardt C., Govers F., Mackerron D.K.L., Taylor M.A., Ross H.A., Eds.; Elsevier Sciences: Amsterdam, Netherlands, 2007; pp. 595-617.
6. Dey P.; Hossain I.; Hossain M.D. Isolation and identification of seed borne *Ralstonia solanacearum* from tomato and brinjal in Bangladesh. *IOSR Jour. of Agri. and Vet. Sci.* **2017**, *10*, 32-39.
7. Siregar B.A.; Giyanto; Hidayat S.H.; Siregar I.Z.; Tjahjono B. Epidemiology of bacterial wilt disease on *Eucalyptus pellita* F. Muell. in Indonesia. *IOP Conf. Ser. Earth Environ. Sci.* **2020**, *468*, 012033.
8. Denny T.P.; Hayward A.C.; *Ralstonia*. In *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd Ed.; Schaad N.W., Jones J.B., Chun W., Eds.; APS Press: St Paul, USA, 2001; pp. 151-173.
9. Sinarmas Forestry. Isolasi dan Identifikasi Penyakit Layu Bakteri pada Eukaliptus. In *Instruksi Kerja R&D PT. Arara Abadi*; R&D PT. Arara Abadi: Perawang, Indonesia, 2015; pp. 4-12
10. Opina N.; Tavner F.; Hollway G.; Wang J.-F.; Li T.-H.; Maghirang R.; Fegan M.; Hayward A.C.; Krishnapillai V.; Hong W. F.; Holloway B.W.; Timmis J. N. A novel method for development of species and strain-specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (Formerly *Pseudomonas solanacearum*). *As. Pac. J. Mol. Biol. Biotechnol.* **1997**, *5*, 19-30.
11. Mafia R.G.; Alfenas A.C.; Penchel F.R.M.; Ferreira M.A.; Alfenas R.F.; Bacterial wilt: pathogen spread and disease effects on the eucalyptus cloning. *Rev Arvore* **2012**, *36*, 593–602.
12. Agarwal V.K.; Sinclair J.B. *Principles of seed pathology*. (2nd ed.); CRC-Press; 1997; 539 pp.
13. Gafur, A. Development of biocontrol agents to manage major diseases of tropical plantation forests in Indonesia: A review. Presented at the 1st International Electronic Conference on Forests (IECF), November 15-30, 2020.
14. Yuliar, Nion Y.A.; Toyota K. Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum*. *Microbes Environ.* **2015**, *30*, 1-11.



© 2020 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (<http://creativecommons.org/licenses/by/4.0/>).