Australasian Plant Dis. Notes (2013) 8:43–47
DOI 10.1007/s13314-013-0092-z

Bacteria associated with sheath browning and grain discoloration of rice in East Timor and implications for Australia's biosecurity

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Received: 27 August 2012 / Accepted: 31 January 2013 / Published online: 15 February 2013

Abstract A survey of rice growing regions in East Timor (Timor Leste) conducted in 2010 has resulted in the identification of several bacterial species associated with sheath browning and seed discolouration. Bacteria were identified by partial sequencing of the 16s rRNA gene and comparison with sequences from GenBank and EZtaxon databases. Bacterial isolates of known pathogens of rice, corn and cotton were identified, along with non-phytopathogenic endophytes. This is an indication that the disease symptoms are likely to be the result of a pathogen complex rather than caused by a single species. Unfortunately, due to current lack of infrastructure in East Timor, definitive pathogenicity testing of these isolates could not be undertaken. However, it is imperative to increase the capacity of East Timorese to improve their diagnostic skills and management of diseases of rice, and ultimately increase productivity. Furthermore, it is important to heighten the awareness of East Timorese farmers of the importance of diseases in rice production. Evidently, there is a potential source of high risk bacterial pathogens in the rice production system in East Timor and this also poses a risk to Australia's biosecurity. Potentially devastating pests and diseases of Australian agriculture currently present in Asia could spread into Australia via East Timor. There are a number of economically important diseases of rice that occur in Southeast Asia which do not currently occur in Australia.

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Keywords Rice pathogen · Endophyte · 16s rRNA · Disease diagnosis

Maize and rice are the main staple foods in East Timor, with an estimated production area of 38,000 ha for rice and 120,000 ha for maize (Worldmark Encyclopedia of Nations 2007). Yield per hectare of rice is approximately 1.5 mt ha, which is low compared to other rice-growing countries in Asia. This can be attributed to poor or non-application of improved varieties, quality seeds, and fertilizer. The lack of managed irrigation also has direct consequences on the number of cropping cycles that can be achieved each year. A further impediment to the yield of rice is the incidence of pests and diseases and the associated lack of crop protection strategies. In 2006, a survey of rice diseases in East Timor was undertaken to ascertain the level of risk posed by the diseases to the Australian rice industry (EH Graham Centre 2006). It highlighted the importance of diseases to rice production in East Timor and heightened the awareness of its local farmers and advisors. The survey also examined East Timorese rice production and key facilities for quarantine and plant disease research. In 2010, rice plant samples displaying sheath browning and grain discoloration were collected from the rice growing regions of East Timor and assessed for the presence of bacteria commonly associated with the disease symptoms.

Rice crops within the villages of Dili, Aileu and Bobonaro Districts (Fig. 1) were visually inspected for sheath browning symptoms and seed discolouration. Symptoms on glumes ranged from necrotic spotting to complete discoloration (Fig. 2). Leaf-sheath lesions were light brown to black, generally irregular in shape resulting in a mottling effect. During the field survey, diseased samples were kept in plastic bags with wet paper towels to provide moisture. In the laboratory, diseased tissues and seeds were surface sterilized in 70 % isopropyl alcohol for 1 min followed by three consecutive rinses with sterile distilled water (SDW) for 1 min each,

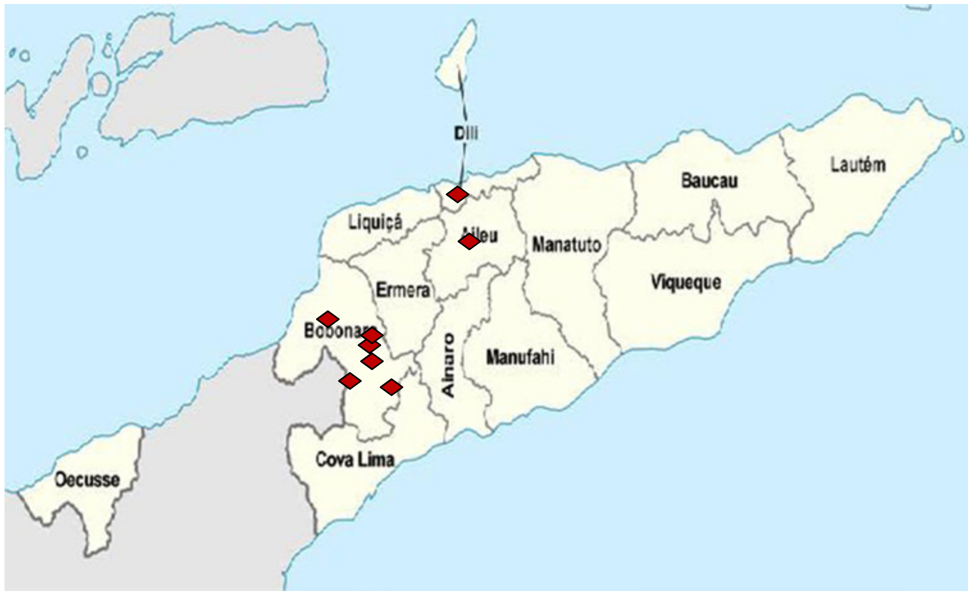

Fig. 1 Diseased sample collection sites in the rice-growing villages of East Timor (Map from http://en.wikipedia.org/wiki/Districts_of_East_Timor)

placed on nutrient agar (NA) and incubated at room temperature for 24 h. Resulting bacterial colonies were selected and suspended in 5 mL SDW. A drop, approximately 50 μL, of each bacterial suspension was applied to Whatman FTA® Classic Cards (Cat. No. WB120065; Whatman International Ltd, Piscataway, NJ, USA) and dried thoroughly. The FTA cards were kept in a sealed plastic bag, stored at room temperature and transported to Australia for processing. Sample preparation of FTA cards was essentially as described in the FTA Cards Data Sheet (Whatman 2007). A single 1.2 mm-disc was punched from each sample using a Harris Uni-Core one-hole punch and mat (Cat. No. WB640001; Whatman International Ltd, Piscataway, NJ, USA) and placed in a microcentrifuge tube. The hole punch was cleaned between samples by rigorous manual wiping with 70 % ethanol. The sample disc was rinsed twice in 150 μL FTA Purification Reagent (Cat. No. WB120204; Whatman International Ltd, Piscataway, NJ, USA), twice in 150 μL TE buffer (10 mM Tris pH7.0: 1 mM EDTA) with shaking at 150 rpm for 3 min. The disc was air dried for 1 h and then used as template for PCR. PCR amplification and direct sequencing of partial 16s rRNA was performed on all samples following the procedure of Cother et al. (2009). Chimera detection and removal was performed using DECIPHER (Wright et al. 2012). The 16 s rRNA sequences were compared to those available in GenBank (Benson et al. 2012) using BLASTN 2.2.26+ (Zhang et al. 2000) and EzTaxon database using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; (Kim et al. 2012) to determine the most likely identities of the isolates.

Fig. 2 Sheath browning and grain discoloration symptoms observed in the rice-growing villages of East Timor on rice cv. IR64

Based on the sequence data a diverse group of bacteria associated with sheath browning and grain discoloration were identified (Table 1). Some of these genera have been previously reported to have caused disease on their respective host, some were constantly associated with the disease and could be possible pathogens. In addition, non-phytopathogenic endophytes and clinical pathogens were also identified. Corresponding DNA sequences were deposited in GenBank with Accession numbers listed in Table 2. Bacterial isolates with sequence similarity for the 16s rRNA of less than 95 % when compared to those in GenBank and EzTaxon databases were excluded from the list. However, such isolates may represent potentially new species. The present result is a clear indication of the difficulties faced when determining the causal organism responsible for the observed disease symptoms. It is likely that the sheath browning and seed discolouration present in a large proportion of rice crops in East Timor is the result of a complex of

Table 1 Identities of bacteria associated with diseased sheath and discoloured rice grains of cv. IR64 in rice-growing villages of East Timor, based on 16 s rRNA sequence

Sample number[1]	Origin (lat; long)[2]	Tissue origin	Colony colour on NA[3]	16 s pairwise identity		Notes
				GenBank	EzTaxon	
DLA64	Fomento, Dili (-8.5549; 125.5670)	sheath	light yellow	*Pseudomonas oryzihabitans*, 97 %	*Pseudomonas oryzihabitans*, 96 %	Rice endophyte (Hardoim et al. 2012)
DLA66	Fomento, Dili	grains	translucent yellow	*Acidovorax avenae*, 98 %	*Acidovorax avenae*, 98 %	Rice pathogen (Schaad et al. 2008)
DLA67	Fomento, Dili	grains	cream	*Burkholderia glumae*, 99 %	*Burkholderia glumae*, 99 %	Rice pathogen (Ham et al. 2011)
DLA68	Fomento, Dili	grains	translucent yellow	*Erwinia sp*., 96 %	*Erwinia sp*.,96 %	Rice pathogen (Goto 1978; Kim and Song 1996)
DLA69	Fomento, Dili	grains	yellow	*Pseudomonas oryzihabitans*, 99 %	*Pseudomonas oryzihabitans*, 99 %	Rice endophyte (Hardoim et al. 2012)
DLA73	Aileu, Aileu (-8.7168; 125.5667)	grains	yellow	*Pantoea stewartii*, 99 %	*Pantoea stewartii*, 98 %	Corn pathogen (Wensing et al. 2010)
DLA74	Aileu, Aileu	sheath	cream	*Acidovorax* sp., 98 %	*Acidovorax* sp., 98 %	Rice pathogen (Schaad et al. 2008)
DLA77	Aileu, Aileu	grains	yellow	*Pantoea ananatis*, 96 %	*Pantoea ananatis*, 97 %	Rice pathogen (Mondal et al. 2011)
DLA78	Aileu,Aileu	grains	light yellow	*Herbaspirillum* sp., 99 %	*Herbaspirillum* sp., 99 %	Rice endophyte (Elbeltagy et al. 2001)
DLA84	Tunu Bibi, Maliana, Bobonaro (-8.9745; 125.1447)	sheath	cream	*Pseudomonas oruzihabitans*, 99 %	*Pseudomonas oryzihabitans*, 99 %	Rice endophyte (Hardoim et al. 2012)
DLA85	Tunu Bibi, Maliana, Bobonaro	grains	yellow	*Rhizobium* sp., *99*%	*Rhizobium* sp., 98 %	Non-phytopathogenic endophyte (Panday et al. 2011)
DLA87	Tunu Bibi, Maliana, Bobonaro	sheath	cream	*Acinetobacter radioresistens*, 98 %	*Acinetobacter radioresistens*, 98 %	Cotton pathogen (Nishimura et al. 1988)
DLA90	Memo, Maliana, Bobonaro (-9.0422; 125.2128)	sheath	yellow	*Pantoea* sp., 98 %	*Pantoea* sp., 99 %	Rice pathogen (Mondal et al. 2011)
DLA94	Raifun, Maliana, Bobonaro (-9.0329; 125.2565)	grains	cream	*Pantoea stewartii*, 99 %	*Pantoea stewartii*, 99 %	Corn pathogen (Wensing et al. 2010)
DLA95	Phoe Dolen, Maliana, Bobonaro (-9.0621; 125.3359)	sheath	cream	*Pseudomonas psychrotolerans*, 99 %	*Pseudomonas psychrotolerans*, 98 %	Clinical pathogen (Hauser et al. 2004)
DLA97	Rita Bou, Maliana, Bobonaro (-9.0478; 125.2647)	grains	cream	*Pantoea stewartii*, 99 %	*Pantoea stewartii*, 99 %	Corn pathogen (Wensing et al. 2010)

[1] Samples collected on 26–28 April 2010 in Dili, Aileu and Bobonaro districts. Bacteria with less than 95 % sequence similarity from both the GenBank and EzTaxon databases were excluded from the list.

[2] Village, sub-district, district (Coordinates: latitude;longitude)

[3] Nutrient Agar

Table 2 GenBank accession numbers for partial 16s rRNA sequences of 16 bacterial isolates reported in this study

Isolate	GenBank accession number
DLA64	JX522461
DLA66	JX522462
DLA67	JX522463
DLA68	JX522464
DLA69	JX522465
DLA73	JX522466
DLA74	JX522467
DLA77	JX522468
DLA78	JX522469
DLA84	JX522470
DLA85	JX522471
DLA87	JX522472
DLA90	JX522473
DLA94	JX522474
DLA95	JX522475
DLA97	JX522476

bacteria rather than a single species. It should be noted also that pathogens other than bacteria (e.g. fungi) can also cause diseases on rice sheaths. To determine which bacteria caused the disease, Koch's postulates need to be performed. Correct disease diagnosis is critical in the choice of disease management strategies. East Timor is ranked as one of the poorest nations in the world, and it is not surprising that the approach of East Timor's farmers and agronomists to the diagnosis and management of disease in their crops is very different from Australia's approach to biosecurity. It is imperative to increase the capacity of the East Timorese to improve disease management, particularly in staple crops, with the aim of ultimately increasing rice productivity. Previous surveys of rice and other agricultural crops in fields and markets of East Timor identified symptoms of a variety of pathogens whose abundance ranged from low to epidemic levels (Ash 2011). Evidently, rice production in East Timor represents a potential source of high risk bacterial pathogens for Australia's rice industry. East Timor is only a short flight from Australia and may act as a stepping stone to a range of pests and diseases from Asia which are potentially devastating for Australian industry. There are a number of economically damaging diseases of rice that occur throughout Southeast Asia which, as yet, do not occur in Australia. Travellers entering Australia often attempt to bring live plants or plant materials that may harbor exotic pests and pathogens. An accidental or intentional introduction of a regulated plant pathogen could easily cause losses to the economy in the tens of billions of dollars. One reason for the success of Australian biosecurity programs has been the implementation of pre-border assessments, particularly in neighbouring countries. The results of this present study not only provide key information essential for the maintenance of pre-border biosecurity and the future protection of the Australian rice industry, but provide essential information for the development of East Timor's own border protection. Associated with hands-on training of plant protection personnel in-country the potential exists for East Timor to improve rice production not only to the level required for self-sufficiency, but also to meet the requirements of world trade.

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