A new shoot and stem disease of *Eucalyptus* species caused by *Erwinia psidii*

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Abstract. A serious disease of green, actively expanding stems of young *Eucalyptus grandis, E. dunnii, E. globulus* and *E. maidenii* has been observed in plantations in Uruguay and Argentina during the course of the past ten years. The symptoms of the disease are unlike those previously observed on any species of *Eucalyptus.* In this study, we describe the symptoms of this new disease and determine its cause. A diagnostic feature of the disease is a red discolouration of the young host tissue and blistering of the young bark leading to rapid shoot death. A bacterium was consistently isolated from the stem blisters on to nutrient agar, purified and a selection of six strains were subjected to

standard phenotypic tests and 16S rRNA-, *gyrB*- and *rpoB*-gene sequencing. The ability of these strains to induce a hypersensitive reaction (HR) was tested on tobacco and a pathogenicity tests were undertaken on a *E. grandis* clone. The bacterium was found to be identical to *Erwinia psidii*. Strains inoculated into tobacco produced a HR within 36 hours and discolouration of internal shoot tissue was observed in the inoculated *E. grandis* clone. *E. psidii* is known to cause die-back of guava (*Psidium guavaja*) which is closely related to *Eucalyptus*, also belonging to the Myrtaceae. Results of this study suggest that *E. psidii* has undergone a host shift to become an important pathogen of *Eucalyptus* spp. that are widely planted in South America to sustain important paper and pulp industries.

Additional keywords: Erwinia psidii, *Eucalyptus grandis*, *Eucalyptus dunnii*, *Eucalyptus maidenii*, *Eucalyptus globulus*, blister bark disease, guava

Introduction

Eucalyptus spp. are extensively propagated in the tropics and southern Hemisphere sustaining important timber and pulp industries with an estimated 18 million hectares planted in 80 countries (FAO 2000). In most of these countries, *Eucalyptus* spp. are non-native and they have consequently been separated from most of their natural enemies (Wingfield *et al*. 2008). However, there are numerous fungal pathogens that have emerged to cause considerable damage to *Eucalyptus* established in plantations. Some of these include *Puccinia psidii* that causes Eucalyptus rust (Coutinho *et al.* 1998; Glen *et al.* 2007) and *Teratosphaeria nubilosa* (Perez *et al,* 2009a; Hunter *et al*. 2009) that causes a serious leaf blotch disease. There are also a number of bacterial pathogens of eucalypts including *Xanthomonas campestris* pv. *eucalypti* (Truman 1974), *Pantoea ananatis* (Coutinho *et al*.

2002) and *X. axonopodis* (Gonçalves *et al*. 2008) that cause leaf blight and die-back as well as *Ralstonia solanacearum* (Dianese *et al*. 1990) that causes bacterial wilt in many tropical countries.

Many *Eucalyptus* pathogens have apparently been introduced into countries where these trees are being grown, together with seeds or other forms of planting stock (Wingfield *et al.* 2008). There are also growing numbers of examples of fungal pathogens that have undergone host shifts from native plants to *Eucalyptus* in areas where they are planted together (Slippers *et al*. 2005). For example, the Eucalyptus rust pathogen *P. psidii*, which affects native Myrtaceae in South America, has become an important pathogen of *Eucalyptus* species on this continent (Coutinho *et al*. 1998; Glen *et al*. 2007). Likewise, numerous members of the Cryphonectriaceae, native on the Melastomataceae in South and Central America have undergone host shifts to cause serious stem canker diseases on *Eucalyptus* (Wingfield 2003; Gryzenhout *et al.* 2006; Gryzenhout *et al.* 2009).

Bacterial plant pathogens typically have a broad host range and in this regard, host shifts are often less obvious than they might be in the case of fungal pathogens. For example, *P. ananatis* not only causes disease in a number of plant species, including *Eucalyptus*, but it has also been recorded as a human pathogen (Coutinho and Venter 2009). In this regard, various bacterial diseases of *Eucalyptus* have emerged in the recent past (Truman 1974; Coutinho *et al*. 2002; Gonçalves *et al*. 2008) and most appear to be native to the countries in which they occur. Although there are no obvious examples of bacterial pathogens moving to *Eucalyptus* from closely related hosts, it is possible that host shifts could occur in the same way that has been true for fungal pathogens.

During the course of *Eucalyptus* disease surveys undertaken in Argentina and Uruguay during the past ten years, a disease previously unknown on *Eucalyptus* was observed on young *E. grandis, E. dunnii, E. globulus* and *E. maidenii* trees. The aim of this study was to describe the disease and to identify its causal agent.

Materials and methods

Symptoms

The earliest symptoms of the disease on young (six months to two-year old) *Eucalyptus* trees are necrotic lesions on newly formed leaves that also often have a halo of bacterial residue around them. The most obvious symptom of the disease is shoot and branch die-back (Fig. 1A). Small stem cankers are present and the wood below these cankers has a light brown discoloration. These symptoms are also associated with blisters below the young actively growing green bark (Fig. 1B) that also often assumes a red colour (Fig. 1C). When punctured, the bark blisters exude copious bacteria. As the disease progresses, cankers develop on the branches and growing shoots (Fig. 1C-F) and these apparently result from the development of opportunistic secondary infections. Isolations from the cankers result in cultures of a *Botryosphaeria* sp. (authors, unpublished) and these fungi are known to be opportunistic pathogens on *Eucalyptus* spp. in Uruguay (Perez *et al*., 2009b).

Isolation from infected tissue

Blisters observed on *E. grandis* and *E. dunnii* stems were carefully punctured with a sterile needle and the exuding bacteria were transferred with a needle to nutrient agar (15g

nutrient broth, 15g agar) in Petri dishes. Petri dishes were incubated for 48 h at 30 ºC. Bacterial colonies were then purified and six strains (BCC 1322, 1325, 1327, 1331, 1334 and 1336) were selected for further study. All strains are maintained in the Bacterial Culture Collection (BCC) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Bacterial characterization

All six purified strains were subjected to Gram staining and the Hugh-Leifson test using Oxidative Fermentative media (Biolab). Genomic DNA was extracted from all strains using the DNeasy™ Blood and Tissue Kit (Qiagen). Almost complete 16S rRNA gene sequences were determined for the six strains using the primers and conditions described by Coenye *et al* (1999). The resulting sequences were compared with those in GenBank using BLAST. In addition, g*yrB* and *rpoB* gene sequences were determined using the primers and conditions described by Brady *et al*. (2008). Consensus sequences from the strains were manually assembled using BioEdit Sequence Alignment Editor v 7.0.9.1 (Hall 1999). Overhangs in the consensus sequences were trimmed after each gene was aligned with the ClustalW multiple alignment tool in BioEdit Sequence Alignment Editor v 7.0.9.1. The best-fit evolutionary model was determined for the 16S rRNA data and for the concatenated data for the *gyrB*- and *rpoB*-genes in Modeltest 3.7 (Posada and Crandall 1998). Maximum likelihood trees were constructed in Phyml (Guidon and Gascual 2003) and bootstrap analysis with 1 000 replicates was performed. *Enterobacter cloacae* and *Pectobacterium carotovorum* ssp. *carotovorum* were selected as outgroups for the 16S rRNA gene- and concatenated phylogenetic trees, respectively.

Pathogenicity tests

Inoculum was prepared by growing each isolate in 50 ml of Nutrient Broth. The flasks were incubated overnight at 28 ºC and the resulting bacteria re-suspended in sterile distilled water. The concentration of the bacterial cells was then adjusted to approximately 10^8 CFU/ml.

In order to determine whether or not the isolates were pathogenic, the appearance of the hypersensitivity reaction (HR) in tobacco (*Nicotiana tabacum* cv samsun) was recorded. The bacterial inoculum was injected into the leaves of the tobacco plants using a 1 ml insulin syringe. The needle was inserted into the main vein and the leaf panels were flooded with the bacterial suspension. A negative control containing only sterile water and a positive control containing the bacterial blight and die-back pathogen, *Pantoea ananatis* (LMG 20103), were also included. Tobacco plants were kept in a greenhouse at approximately 26 ºC with natural day and night light cycles. The plants were assessed after 24, 48 and 36 hours for the development of a HR. A positive HR response was recorded when a complete and rapid collapse of the inoculated leaf tissue or light brown necrosis of the water soaked tissue occurred within 36 hours of inoculation.

Inoculum was prepared in the same manner as for the HR tests and used to inoculate actively growing green stems of a 10 two-year old *E. grandis* clone. A 1 ml insulin syringe needle was carefully inserted beneath the bark and approximately 0.1 ml of either the inoculum or sterile water was injected into the tissue. Inoculated plants were covered with plastic bags in order to maintain high humidity. Bags were removed after seven days and the inoculated stems were assessed for disease development every 24 hours for a further

period of 7 days. Plants were kept at 26 ºC with natural day/night light cycles. Isolations on Nutrient Agar were made from lesions that developed on the inoculated stems. In order to confirm the identity of the re-isolated bacteria, 16S rRNA gene sequences were generated for them and these were compared with those of the inoculated bacteria.

Results

Bacterial characterization

All six strains used in this study had rod-shaped and Gram negative cells. They were also able to utilize glucose both fermentatively and oxidatively. These results suggested that the strains belonged to the family *Enterobacteriaceae*. The 16S rRNA gene sequences of all the strains isolated from blisters on the *Eucalyptus* stems had 100% homology to the sequences for *Erwinia psidii*. These strains also clustered with reference strains of *E. psidii* in the phylogenetic trees based on the 16S rRNA (figure not shown) and concatenated sequences for the *gyrB*- and *rpoB*-genes (Fig. 2). These clusters were supported by bootstrap values of 100 %, confirming the identity of the strains. The GenBank numbers for the *gyrB*- and rpoB- genes are as follows: GU991637, GU991643 (BCC 1322), GU991638, GU991644 (BCC 1325), GU991639, GU99165 (BCC 1327), GU991640, GU 99166 (BCC 1331), GU991641, GU99167 (BCC 1334) and GU991642, GU99168 (BCC 1336).

Pathogenicity tests

Pantoea ananatis and the six strains isolated from the blisters occurring on *E. grandis* and representing *E. psidii* produced a hypersensitive reaction on tobacco leaves 24 hours after

inoculation. In contrast, the leaves treated with sterile distilled water showed no symptoms.

Blisters typical of the disease found in the field on young *E. grandis* stems did not form on stems inoculated with strains of *E. psidii*. However, stem tissue at the point of inoculation and below the sites of inoculation was distinctly discoloured similar to that seen in natural infections. After 14 days, the lesions extended at least 1 cm from the point of inoculation. No symptoms developed in the plants inoculated with the sterile distilled water. *E. psidii*, identified using DNA sequence comparisons, was re-isolated from the margins of the lesions on the inoculated plants but not from the controls.

Discussion

This study describes a previously unknown shoot and stem die-back disease observed on the shoot and branches of young *Eucalyptus* trees in Argentina and Uruguay. Bacterial infections are most closely associated with symptomatic tissue and the isolated bacterium was identified as *Erwinia psidii*. This is the first report of an *Erwinia* species causing disease symptoms in *Eucalyptus* trees. *E. psidii* was first described in 1987 in Brazil where it caused dieback on *Psidium guajava* (guava trees) (Neto *et al*. 1987). On *P. guajava*, the pathogen infects branches and twigs and causes collapse of the vascular tissue and die-back. It is currently one of the most important pathogens affecting guava in central Brazil (Texeira *et al*. 2009) and results of the present study suggest that *E. psidii* has undergone a host shift to infect *Eucalyptus* spp.

Neto *et al*. (1987) inoculated several members of the Myrtaceae with *E. psidii*, including *Eucalyptus citriodora*. Inoculation was done by pricking the young stems with a dissecting needle immersed in a bacterial suspension. From this host range study, they concluded that only strawberry guava (*Psidium cattleianum*), *Eugenia jambolana* and *Melaleuca* spp. are susceptible hosts. *Eucalyptus* was not considered as a host of this pathogen at that time. But *E. citriodora* tested by Neto *et al.* (1987) is a species very different to those affected by *E. psidii* in Uruguay and Argentina and an inoculation to it would not be expected to reflect susceptibility of all *Eucalyptus* spp.

Pathogenicity tests on *Eucalyptus* undertaken in this study resulted in distinct cambial lesions similar to those found on young *Eucalyptus* stems in the field. Isolation of *E. psidii* from the lesions provided robust evidence that the bacterium is the cause of the disease of *Eucalyptus* discovered in this study. The unusual blisters that are sometimes found on the very young bark of stems and branches did not develop in the pathogenicty tests. This could be due to a number of factors including environmental conditions, genotype of the plants inoculated or the age of the inoculated tissue, which is difficult to simulate in artificial inoculations.

The fact that *E. psidii* has now been found as a pathogen of *E. grandis* suggests that this bacterium has undergone a host shift to *Eucalyptus* from the related native *P. guajava*. This adds to a number of important and relatively host-specific pathogens that have adapted to infect *Eucalyptus* where these trees are planted as non-natives. Some of the more prominent examples include species of *Chrysoporthe* that have moved from native Myrtaceae in Africa and South America to cause cankers on *Eucalyptus* (Rodas *et al*. 2005) and the *Eucalyptus* rust complex (Coutinho *et al*. 1998; Glen *et al*. 2007). Most of the

reported cases have been of fungal pathogens and *E. psidii* represents the first clear example of a host-specific bacterial pathogen undergoing a host shift to *Eucalyptus*.

The disease of *Eucalyptus* caused by *E. psidii* described in this study appears to be restricted to trees in the first two years of growth and particularly to young, rapidly expanding tissues. Where tops of trees are killed, the disease appears to be exacerbated by secondary infections by *Botryosphaeria* spp. These fungi are well known endophytes on *Eucalyptus* that cause disease problems, typically associated with stress (Slippers and Wingfield 2007). *Botryosphaeria* spp. are also well–known pathogens of *Eucalyptus* in Uruguay (Perez *et al.* 2008; 2010) and they clearly appear to contribute to the damage observed on stems infected by *E. psidii*.

While infections due to *E. psidii* can cause relatively serious damage to young trees, especially through the death of tree tops and the development of double leaders, the trees also appear to recover relatively rapidly. This is probably due to their very rapid growth and the disease has not been seen on older trees, particularly not those that have grown beyond the point where lower branches have been shed and where humidity in the stands is consequently lower. In this respect, the disease does not appear to be a serious threat to *Eucalyptus* spp. although it should be monitored carefully in the future.

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Figure legends

Fig. 1 Field symptoms of the disease on *Eucalyptus* caused by *E. psidii*

A Shoot tip dieback of a young *E. grandis* clone

B Weakened stem due to infection which led to breakage

C Blisters on a *E. grandis* stem

D Stem canker on young, actively growing *E. grandis* tissue

E Advanced stem canker

F After removal of the bark, discoloured tissue is evident which is the result of both *E. psidii* and endophytic *Botryosphaeria* infections

Fig. 2 Maximum likelihood tree based on the concatenated nucleotide sequences of *gyrB* and *rpoB* genes. Bootstrap values after 1000 replicates are expressed as percentages. *Pectobacterium carotovorum* ssp. *carotovorum* was included as an outgroup. The scale bar indicates the fraction of substitutions per site

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