



# *Xanthomonas perforans*: a tomato and pepper pathogen associated with bacterial blight and dieback of *Eucalyptus pellita* seedlings in Indonesia

K. N. Bophela<sup>1,2</sup> · S. N. Venter<sup>1,2</sup> · M. J. Wingfield<sup>2</sup> · A. Duran<sup>3</sup> · M. Tarigan<sup>3</sup> · T. A. Coutinho<sup>1,2</sup> 

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## Abstract

Leaf and shoot blight, often accompanied by die-back symptoms, on *Eucalyptus* species, hybrids and clones have been reported from a number of countries. More than one bacterial species has been found to cause these symptoms. In this study, a leaf disease of *E. pellita* in Indonesia was investigated. The disease was found primarily on nursery plants and young trees that recovered within the first year of growth. Leaf samples were collected from symptomatic trees, and isolations consistently yielded a *Xanthomonas* sp. Sequencing of the 16S rRNA gene region and multilocus sequence analysis (MLSA) was performed on 19 of the 61 *Xanthomonas* isolates obtained. In the MLSA, four genes, namely, *dnaK*, *fyuA*, *gyrB* and *rpoD*, were sequenced and the isolates were identified as *X. perforans*. Four representative isolates, at a concentration of 10<sup>6</sup> CFU/ml, were leaf-infiltrated and spray-inoculated on to *E. pellita*, tomato and pepper seedlings. The type isolate of *X. perforans* was included in the pathogenicity trials as a positive control. All four isolates of *X. perforans*, inclusive of the type isolate, induced bacterial spot symptoms on tomato and pepper seedlings. They also caused water-soaked lesions on the leaves of *E. pellita* seedlings, characteristic of the symptoms observed in the field. This is the first report of *X. perforans* infecting leaves of a woody host.

**Keywords** *Xanthomonas perforans* · *Eucalyptus pellita* · Bacterial blight · Tomato · Pepper

## Introduction

Blight and dieback symptoms on *Eucalyptus* can be caused by a number of bacterial species in different parts of the world. In Australia, *Xanthomonas dyei* pv. *eucalypti* (*X. campestris* pv. *eucalypti*) caused leaf blight in eucalypts (*Corymbia citriodora*) in the 1970s (Truman 1974). In Brazil and Uruguay, the disease, accompanied by dieback, is caused by *X. axonopodis* pv. *eucalyptorum* (Ferraz et al. 2018; Gonçalves et al. 2008). *Pantoea ananatis* (Coutinho et al. 2002) and *X. vasicola* (Coutinho et al. 2015) are responsible for the disease on *Eucalyptus* in South Africa. Although not specifically a leaf

pathogen, *Erwinia psidii* infects the vascular tissues of young shoots and, leaves that can result in severe dieback in young *Eucalyptus* plantations (Arriel et al. 2014; Coutinho et al. 2011).

In earlier studies, *Xanthomonas* species have been identified based on phenotypic and biochemical properties (Truman 1974; Van den Mooter and Swings 1990). This led to considerable taxonomic confusion and misidentification of bacterial isolates. Other methods used included repetitive sequence PCR (rep-PCR), amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP), which showed high similarities to the genomic clusters described by Rademaker et al. (2000); and Schaad et al. (2005); Vauterin et al. (1995). Observations from single gene-based phylogenies also produced corresponding group homologies (Hauben et al. 1997; Parkinson et al. 2007). Although these techniques were consistent in producing homologous groupings, certain limitations made them unsuitable for routine diagnosis of *Xanthomonas*. One of these limitations was the apparent difficulty in comparing and standardizing gel profiles between laboratories. Additionally, a lack of resolution of single gene phylogenies was also observed within species. Consequently, a multilocus sequence analysis (MLSA)

✉ T. A. Coutinho  
teresa.coutinho@up.ac.za

<sup>1</sup> Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Private Bag X28, Pretoria 0002, South Africa

<sup>2</sup> Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Private Bag X28, Pretoria 0002, South Africa

<sup>3</sup> AAA R&D, PT. Riau Andalan Pulp and Paper, Pangkalan Kerinci, Pelalawan, Riau 28000, Indonesia

(Young et al. 2008, 2010) has been used to produce a classification framework using two or more protein-coding genes located on the core genome (Gevers et al. 2005).

The aims of this study were i) to identify the causal agent of a leaf disease that is common on young *Eucalyptus pellita* trees in Indonesian plantations and ii) to test the pathogenicity of the representative isolates of the putative pathogen on *E. pellita* seedlings.

## Materials and methods

### Bacterial isolates

Leaves of young *E. pellita* trees between 1 and 6-months-old in plantations in Riau, South Sumatra, Indonesia, commonly displayed symptoms of a leaf disease reminiscent of bacterial infection. Symptoms included necrotic tissue typically surrounded by distinct chlorotic zones (Fig. 1). The symptoms were common on nursery plants prior to outplanting and persisted during the first 6 months of growth. Subsequently, the infected leaves typically abscised from the trees that recovered and grew in the absence of symptoms.

Symptomatic leaves were randomly collected from different trees widely distributed in plantations and nurseries in South Sumatra. Although these did not form part of a systematic survey, the collections represented a wide distribution of

the symptoms in the sampled area. Isolations for bacteria were made from the affected tissues. Diseased tissue was submerged into a 10% (v/v) sodium hypochlorite solution for 1 min and then into 70% (v/v) ethanol for a further minute before being crushed in a sterile mortar containing 1 mL sterile 10 mM phosphate buffer with a pestle. The resulting suspension was streaked on to nutrient agar (NA) (Biolab Diagnostics, Merck, South Africa) and incubated at 28 °C for 48 h. Single colonies were purified. Care was taken to analyse samples from different areas and trees separately. Sixty-one pure cultures of suspected *Xanthomonas* strains were deposited in the Bacterial Culture Collection (BCC) in the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

### DNA extraction

Single colonies of each isolate were suspended in 1 M phosphate buffer solution [4.0 g NaCl, 0.1 g KCl, 0.72 g Na<sub>2</sub>HPO<sub>4</sub> and 0.12 g KH<sub>2</sub>PO<sub>4</sub> dissolved in 500 mL distilled water; pH 8] and genomic DNA was extracted using the Zymo Research Genomic DNA™ Tissue Miniprep Kit (Inqaba Biotech, South Africa) following the manufacturer's instructions. The quantity and quality of the DNA were measured using a spectrophotometer, NanoDrop™ 2000 (Thermo Scientific, USA), and gel electrophoresis, respectively.

**Fig. 1** Typical symptoms of bacterial blight on *Eucalyptus pellita* a) Leaf necrosis surrounded by chlorotic zones b) Enlarged view of leaf necrosis surrounded by chlorotic zones c) Bacterial blight symptoms on young *E. pellita* seedlings as seen in a nursery



## 16S rRNA sequencing

The 16S rRNA region for each DNA template was amplified using universal primers described previously by Coenye et al. (1999). The partial sequences were manually edited using Chromas LITE Sequence Alignment Editor v2.1.1 (Technelysium Pty Ltd., Australia). The sequences were submitted through the BLAST search engine and compared against reference sequences in the GenBank database (National Center for Biotechnology Information, USA). Thereafter, the sequences of the isolates and reference/type isolates of *Xanthomonas* species obtained using GenBank accession numbers from Hauben et al. (1997) were uploaded on to the MAFFT online sequence alignment program v7.0 (Kato et al. 2002; Kato and Standley 2013) to verify sequence orientation and also to perform a multiple sequence alignment. The aligned sequences were exported to BioEdit Sequence Alignment Editor v7.0.9.0 (Hall 1999) and the overhangs trimmed. A best fit evolutionary model was determined using JModeltest v2.0 (Posada 2008; Posada and Crandall 1998) and a maximum likelihood analysis performed using PhyML v3.0 (Guindon et al. 2010; Guindon and Gascuel 2003). The Hasegawa-Kishino-Yano (HKY) model was chosen for the maximum likelihood analysis with rate variation among sites (+G) (Hasegawa et al. 1985). A bootstrap analysis

of a 1000 replicates was performed in order to determine confidence in branching points.

## Multilocus sequence analysis (MLSA)

Nineteen *Xanthomonas* isolates were selected for further identification (Table 1) and subjected to a multilocus sequence analysis (MLSA) using primer sets of four house-keeping genes, namely, *dnaK*, *fyuA*, *gyrB* and *rpoD*, and cycle conditions described by Young et al. (2008). The cycle conditions for the sequencing reactions were as previously described by Coenye et al. (1999). The sequencing products were precipitated using the sodium acetate-ethanol protocol, and the samples were submitted to the DNA sequencing facility at the University of Pretoria, South Africa and sequenced using the ABI3130XL sequencer (Life Technologies, USA).

The partial sequences of the four protein-coding genes were analysed as mentioned previously. The sequences of the isolates were aligned with the reference/type isolates of *Xanthomonas* spp., obtained from Professor CT Bull, Pennsylvania State University, USA. The partial sequences of the four protein-coding genes *dnaK*, *fyuA*, *gyrB* and *rpoD* for the *Xanthomonas* isolates and reference sequences of type species were trimmed to nucleotide lengths of 769, 524, 516 and 701 nt, respectively. Best fit evolutionary models were

**Table 1** List of proposed *Xanthomonas* strains used in the study obtained from Indonesia and Genbank accession numbers (where available)

Isolate	Host	Year	<i>dnaK</i> *	<i>gyrB</i> *	<i>rpoD</i> *	<i>fyuA</i> *
BCC972	<i>Eucalyptus</i> sp.	2007	MK844139	MK844177	MK844158	MK844196
BCC975	<i>Eucalyptus</i> sp.	2007	MK844140	MK844178	MK844159	- <sup>a</sup>
BCC977	<i>Eucalyptus</i> sp.	2007	MK844141	MK844179	MK844160	MK844197
BCC978	<i>Eucalyptus</i> sp.	2007	MK844142	MK844180	MK844161	- <sup>a</sup>
BCC980	<i>Eucalyptus</i> sp.	2007	MK844143	MK844181	MK844162	MK844198
BCC982	<i>Eucalyptus</i> sp.	2007	MK844144	MK844182	MK844163	MK844199
BCC1284	<i>Eucalyptus</i> sp.	2012	MK844145	MK844183	MK844164	MK844200
BCC1285	<i>Eucalyptus</i> sp.	2012	MK844146	MK844184	MK844165	MK844201
BCC1286	<i>Eucalyptus</i> sp.	2012	MK844147	MK844185	MK844166	MK844202
BCC1297	<i>Eucalyptus</i> sp.	2012	MK844148	MK844186	MK844167	MK844203
EPK001	<i>E. pellita</i>	2015	MK844149	MK844187	MK844168	MK844204
EPK004	<i>E. pellita</i>	2015	MK844150	MK844188	MK844169	- <sup>a</sup>
EPK006	<i>E. pellita</i>	2015	MK844151	MK844189	MK844170	MK844205
EPK008	<i>E. pellita</i>	2015	MK844152	MK844190	MK844171	MK844206
EPK015	<i>E. pellita</i>	2015	MK844153	MK844191	MK844172	MK844207
EPK018	<i>E. pellita</i>	2015	MK844154	MK844192	MK844173	MK844208
EPK027	<i>E. pellita</i>	2015	MK844155	MK844193	MK844174	- <sup>a</sup>
EPK028	<i>E. pellita</i>	2015	MK844156	MK844194	MK844175	- <sup>a</sup>
EPK043	<i>E. pellita</i>	2015	MK844157	MK844195	MK844176	MK844209

<sup>a</sup> Sequence not available for bacterial isolate

BCC = Bacterial Culture Collection, Forestry and Agricultural Biotechnology Institute, PTA, South Africa

\* = GenBank accession numbers



determined, and maximum likelihood analyses performed for all four protein-coding genes (data not shown) and concatenated dataset (Fig. 2). *Stenotrophomonas maltophilia* ICMP17033<sup>T</sup> was chosen as the outgroup taxon for these analyses with the exception of the *fyuA* phylogeny (data not shown). The chosen models for the concatenated dataset were *GTR* + *I* + *G*, *TIM2* + *I* + *G* for *dnaK* and *fyuA*, *TIM1* + *G* for *gyrB*, and *TIM3* + *I* + *G* for *rpoD*. Bootstrap analyses of 1000 replicates were performed for each dataset to define confidence in branching points.

## Pathogenicity trial

### Inoculum preparation

Four isolates (EPK8, EPK15, EPK28 and EPK43) were selected and grown on NA and incubated for 48 h at 28 °C. Single colonies obtained from the pure cultures were then suspended in 25 mL nutrient broth (NB) (Biolab Diagnostics, Merck, South Africa) and the samples were placed in a shaking incubator at 120 rpm for 18 h at 28 °C. The bacterial suspensions were adjusted to a concentration of 10<sup>6</sup> cfu/mL (OD<sub>600</sub> = 0.01) using 10 mM phosphate buffer solution.

### Seedling inoculations

*Eucalyptus pellita* seeds were cultivated in a 12.5 cm pot with potting soil 60 dm<sup>3</sup> (Garden Master, South Africa (SA)), vermiculite 15 dm<sup>3</sup> (Bark Unlimited, SA) and river sand mixture (2:1:1), and kept in a phytotron at approx. 25 °C with 16 h day and 8 h night conditions. The emerging *E. pellita* seedlings were transplanted into small plastic bags using the same soil mixture described above. The nutrients were supplemented using organic fertiliser containing 80 g/kg N, 20 g/kg P, 58 g/kg K (4:1:3) (Nitrosol, Efekto, SA) once each week before seedling emergence, and subsequently once bi-weekly. The transplanted seedlings were maintained under the same greenhouse conditions mentioned above. The *Solanum lycopersicum* (tomato) cv. ‘Moneymaker’ and *Capsicum annum* (pepper) cv. ‘Jupiter’ seedlings were sourced from a nursery (Sunshine seedlings, Pietermaritzburg, SA) and maintained under the same greenhouse conditions as for the *E. pellita* seedlings.

Fifteen seedlings each of 1-year-old *Eucalyptus pellita*, tomato cv. Moneymaker and pepper cv. Jupiter, both 6 weeks old, were used in the inoculation trial with three replications per isolate. Prior to the inoculations, the seedlings were placed in plastic square containers, respective to the isolate used for inoculation, and arranged inside the phytotron following a randomised design. Plants were inoculated with one of the four isolates of *Xanthomonas perforans*. *Pantoea ananatis* LMG20103 was used as the positive control for the *E. pellita* inoculations and sterile phosphate buffer as the

negative control. *Xanthomonas perforans* ICMP16690<sup>T</sup> was included as a positive control for the inoculation of tomato and pepper seedlings.

For each seedling, 2–3 leaves were infiltrated with the bacterial suspension at multiple sites along the main vein, flooding the leaf panels, using a 1 mL blunt-end insulin syringe in the case of *E. pellita*. Tomato and pepper leaves were spray-inoculated on both the abaxial and adaxial sides until run-off. The plants were covered with plastic bags to induce a humid environment for 48 h and maintained in an enclosed phytotron at approximately 26 °C with 16 h day and 8 h night cycles. After 48 h, the plastic bags were removed and the plants were monitored for development of disease symptoms. The presence or absence of typical water-soaked lesions on inoculated plants was recorded every 24 h for 1 month. The inoculation trial was repeated once, but in the second trial, the plants of *E. pellita* were spray-inoculated as opposed to infiltration with the bacteria.

### Re-isolation procedure

A subset of inoculated leaves showing leaf spot symptoms, and leaves inoculated with 10 mM phosphate buffer, were collected and surface sterilized by submersion into a 10% (v/v) sodium hypochlorite solution for 1 min and then into 70% (v/v) ethanol for a further minute. The leaves were rinsed with sterile distilled water three times, and 1 mm<sup>2</sup> leaf pieces from the leading edge of lesions were macerated in 10 mM phosphate buffer solution and 30 µL of the resulting suspension streaked on to NA. The plates were incubated at 28 °C for 48 h.

### Identification of recovered bacterial isolates

Genomic DNA from single colonies of bacterial cultures was extracted using the ZR Genomic DNA<sup>TM</sup> Tissue Miniprep Kit following the manufacturer’s instructions. The small ribosomal sub-unit gene region of the recovered isolates was sequenced using PCR based techniques mentioned previously. In the case of a positive 16S rRNA sequence identity for *Xanthomonas*, the *rpoD* gene region was sequenced.

## Results

### 16S rRNA sequencing and multilocus sequence analysis (MLSA) classification

The results of the BLAST search across the GenBank database revealed a 99% sequence similarity with *Xanthomonas* (61), *Pantoea stewartii* (1) and *Pseudomonas oryzae* (1). Only the *Xanthomonas* strains were further analysed. These



**Fig. 2** A Maximum Likelihood tree representing the concatenated phylogeny of the *Xanthomonas* isolates and reference isolates. Bootstrap values after 1000 replicates are indicated as percentages (only values 50 and above are shown). *Stenotrophomonas maltophilia* ICMP17033<sup>T</sup> was

included as an outgroup. The scale bar represents the number of substitutions per site. Isolates marked with an asterisk (\*) represent sequences originally from the study by Ferraz et al. 2018. Postscript 'T' refers to type isolate of the species.

**Table 2** Pathogenicity trial results of *Xanthomonas perforans* isolates infiltrated into leaves of *Eucalyptus pellita* seedlings recorded for a period of 9 days post inoculation

Isolate ID	Disease symptom progression after 48 h incubation*						
	3dpi	4dpi	5dpi	6dpi	7dpi	8dpi	9dpi
Control <sup>a</sup>	–	–	–	–	–	–	–
EPK008 <sup>b</sup>	–	+	++	++	++	++	++
EPK015 <sup>b</sup>	–	+	+	+	+	+	+
EPK028 <sup>b</sup>	–	+	++	++	++	++	++
EPK043 <sup>b</sup>	–	+	++	++	++	++	++
ICMP16690 <sup>T</sup>	–	–	–	+	+	+	+
LMG20103	–	+	+	+	+	+	+

<sup>a</sup> 10 mM phosphate buffer<sup>b</sup> Bacterial concentration at 10<sup>6</sup> CFU/ml, postscript T = Type isolate of the species

\*Observations shown until 9 days post inoculation as no differences in symptoms were seen thereafter, – No lesion, + Water-soaked lesion, ++ Necrotic lesion, +++ Expanded necrotic lesion

represented different sampled areas in Indonesia and spanning different periods of sample collection.

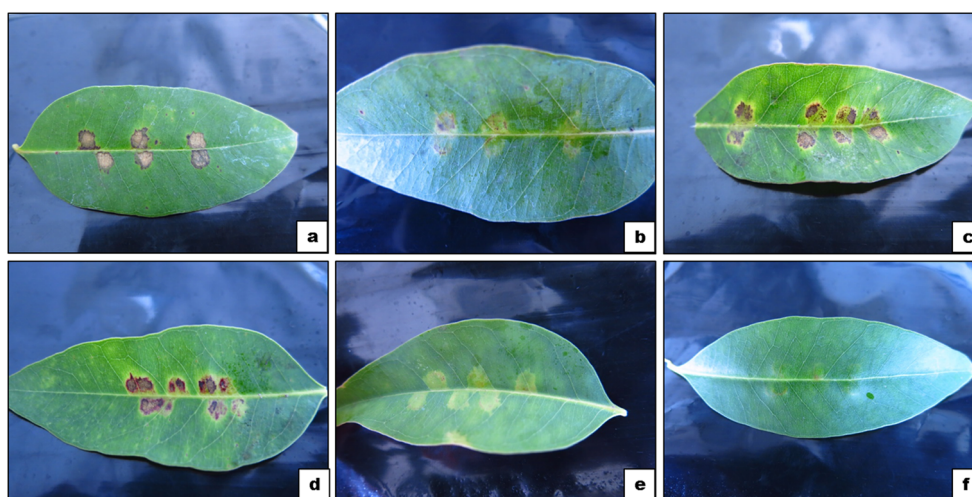
The four protein-coding gene datasets, namely, *dnaK*, *fyuA*, *gyrB*, and *rpoD*, were used to generate a concatenated dataset. Congruency was tested using the four sequence datasets and based on the results of the partition-homogeneity test ( $p > 0.05$ ) the datasets were concatenated. The nucleotide sequence for the combined dataset was 2510 nt in length. The concatenated phylogeny (Fig. 2) revealed a clustering of the *E. pellita* isolates from Indonesia with *Xanthomonas perforans*. The topology of this clade was consistent with the observations of the individual gene phylogenies (data not shown).

Sub-clustering was observed between members of *X. perforans* which may be indicative of the presence of potential sub-populations within this species. The Indonesian isolates formed a discrete sub-group separate from the reference isolate, *X. perforans* ICMP109, the type isolate, *X. perforans* ICMP16690 and isolates LPF581, LPF583, LPF596, LPF597 and LPF598 obtained from symptomatic leaves of *Eucalyptus urophylla* x *E. globulus* clones in Brazil (Fig. 2).

### Pathogenicity trial

Symptom development was slow in the early stages of infection on *E. pellita* seedlings (Table 2). However, at 5 days post-inoculation (dpi), water-soaking was observed on leaves of seedlings infiltrated with EPK28 and EPK43. A mild to weak reaction was observed on *E. pellita* leaves infiltrated with EPK8, EPK15, *X. perforans* ICMP16690<sup>T</sup> and *P. ananatis* LMG20103. No reaction was observed for the negative control. By 18 dpi, the water-soaked lesions had expanded into reddish-brown necrotic lesions surrounded by chlorosis resembling the symptoms observed in the field (Fig. 3). *Eucalyptus* seedlings inoculated with *X. perforans* ICMP16690<sup>T</sup> became chlorotic at the site of inoculation, indicative of a typical HR reaction caused by a non-host pathogen (Fig. 3e). These observations were consistent throughout the period of the pathogenicity trial. However, the lesions on spray-inoculated *E. pellita* seedlings were extremely small, less than 2 mm in size (data not shown).

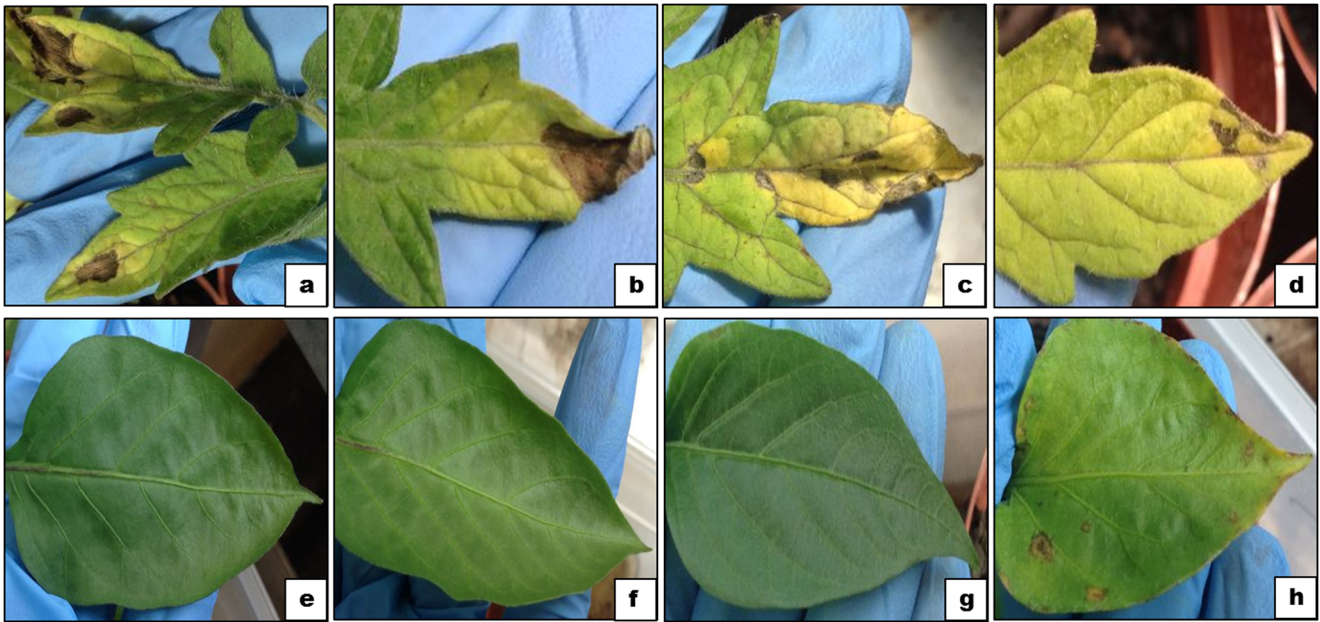
Characteristic leaf spot symptoms were observed on tomato seedlings from 3 dpi onwards, inoculated with the three representative *Xanthomonas perforans* isolates, including *X. perforans* ICMP16690<sup>T</sup> (Fig. 4). In



**Fig. 3** Symptoms caused by *Xanthomonas perforans* infiltrated into 1 year old *Eucalyptus pellita* leaves. **a)** Brown necrotic lesions caused by EPK43; **b)** faint red-brown chlorotic lesions caused by EPK15; **c)** dark red-brown lesions surrounded by chlorosis caused by EPK8 and **d)** EPK28; **e)** chlorotic lesions caused by *Xanthomonas perforans* type

isolate, ICMP16690<sup>T</sup>. **f)** A mild hypersensitive reaction was observed on *E. pellita* leaves infiltrated with *Pantoea ananatis* LMG20103. Pictures were taken at 18 days post-inoculation. Postscript 'T' refers to type isolate of the species





**Fig. 4** Symptoms caused by *Xanthomonas perforans* spray-inoculated on leaves of six-week-old *Solana lycopersicum* (tomato) cv. ‘Moneymaker’ and *Capsicum annum* (pepper) cv. ‘Jupiter’ seedlings. On tomato seedlings, **a**) necrotic lesions surrounded by chlorosis caused by EPK8; **b**) necrotic lesions resulting in curling at the leaf tip and chlorosis caused by EPK28; **c**) chlorotic and marginal necrotic lesions caused by EPK43 and **d**) the type isolate, ICMP16690<sup>T</sup>. **e**) No symptoms were observed on

pepper leaves spray-inoculated with EPK8 and **f**) EPK28; **g**) a single necrotic lesion on the leaf margin caused by EPK43. And **h**) necrotic lesions surrounding the leaf margin and on the leaf lamina caused by the type isolate of *Xanthomonas perforans*, ICMP16690<sup>T</sup>. Pictures were taken 4 days post-inoculation. Postscript ‘T’ refers to type isolate of the species

contrast, only one of the representative isolates (EPK43), and the type strain (ICMP16690<sup>T</sup>) had caused leaf spots on pepper seedlings, albeit weak, by the time of termination of the trial (Fig. 4g, h). Similar results were observed for the tomato and pepper seedlings spray-inoculated with the three representative *X. perforans* isolates in the second pathogenicity trial (data not shown). Leaf spots were absent from the negative controls of all three plant species.

*Xanthomonas perforans* was recovered from all of the *E. pellita*, tomato and peppers samples selected for re-isolation.

## Discussion

This study identified the causal agent of a commonly occurring leaf disease of *Eucalyptus pellita* in nurseries and plantations in Indonesia as *X. perforans*. To the best of our knowledge, this is the first report of *X. perforans* causing a leaf disease on a woody host. *Xanthomonas perforans* is a well-known pathogen causing bacterial leaf spot of tomato and pepper in many parts of the world (Jones et al. 1995; Stall et al. 1994; Vicente et al. 2006). Its ability to cause disease symptoms in *Eucalyptus pellita* was surprising and suggests a host

shift (Coutinho et al. 2015; Wingfield et al. 2008) possibly from tomato or pepper plants grown in the region.

The disease of *E. pellita* considered in this study appears to be of relatively minor importance. The symptoms can first appear in young nursery plants and persist when trees are established in plantations. This can result in the loss of young leaves that appears to slow establishment, but the infected leaves are lost during the first few months of tree growth, which is typically very rapid reaching up to 3 m in the first 6 months. By that time, most infected leaves have been lost, and trees grow free of symptoms.

The origin of infection of *E. pellita* plants by *X. perforans* is unknown, but the fact that seedlings first display typical symptoms as they mature in the nursery suggests that infection originates at that time. Infection possibly occurs via contaminated irrigation water and/or from seed, which could be internally infected with the bacterium. *Xanthomonas perforans* is known to be internally seedborne in both tomato and pepper (Jones et al. 1986). These questions require further study and their understanding should help to manage the problem. The fact that tomato plants were shown to be susceptible to infection by *X. perforans* in this study might suggest that the bacterium originated on tomato but that question requires further investigation.

The phylogenies of the *Xanthomonas* isolates from *E. pellita* based on the four protein-coding genes, and concatenated dataset were congruent, in most cases, for the

main clusters investigated in this study. Overall, although highly supported by all phylogenies, the taxonomic position of the Indonesian isolates relative to the type isolate, *X. perforans* ICMP16690, remained ambiguous, with these isolates residing in a sub-lineage within the *X. perforans* cluster (Fig. 2). A MLSA phylogeny constructed with a nucleotide dataset contains a high level of substitution saturation, homoplasy and codon bias (Palmer et al. 2017). However, protein-coding genes are still widely used as phylogenetic markers in numerous research studies for routine diagnosis and taxonomic purposes (Coutinho et al. 2015; Parkinson et al. 2007; Rodriguez-R et al. 2012; Young et al. 2008, 2010). Taken collectively, in order to determine if there is indeed a subpopulation within this cluster, a true phylogeny will need to be reconstructed using core-genomic data. The taxonomic position of *X. alfalfae* ssp. *alfalfae* ICMP5718, shown in the concatenated phylogeny presented in this study needs clarification. The concatenated phylogeny suggests that this species was incorrectly identified as an *X. alfalfae* strain while it is, in fact, *X. perforans*, therefore, making MLSA approach a robust classification tool for identifying known and unknown isolates up to but not limited to species level.

Bacterial diseases of *Eucalyptus* spp. have become important as these trees are increasingly grown in plantations globally. The leaf disease on *E. pellita* caused by *X. perforans* is one of a number of bacterial leaf diseases of *Eucalyptus* spp. including those caused by *X. axonopodis* (Gonçalves et al. 2008), *P. ananatis* (Coutinho et al. 2002), and *X. vasicola* (Coutinho et al. 2015). These diseases are generally not considered to be serious with damage mostly occurring in seedlings during propagation and in the early stages of plantation development. This is in contrast to more serious problems associated with bacteria such as two *Ralstonia* spp., *Ralstonia solanacearum* and *R. pseudosolanacearum* (Carstensen et al. 2017; Coutinho et al. 2000; Dianese and Dristig 1993) that are associated with wilt of *Eucalyptus* in various parts of the world and *Erwinia psidii* (Arriel et al. 2014; Coutinho et al. 2011) that causes a serious wilt disease in young plantations in South America. These diseases add to growing challenges facing *Eucalyptus* plantation forestry globally (Wingfield et al. 2008).

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## Compliance with ethical standards

**Conflict of interest** No conflict of interest exists.

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