

Infectivity Assays and Sequence Analyses for Unassigned *Pseudomonas* Species as Putative Cause of Dieback Disease of *Dalbergia sissoo* Roxb. in Bangladesh

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Abstract

The causal agent(s) of dieback of sissoo (*Dalbergia sissoo* Roxb.) have not yet been identified unequivocally. Putative microbial pathogens (fungi and bacteria) were studied in dieback affected sissoo trees collected from Bangladesh, using plant pathological techniques combined with molecular tools. DNA based characterization showed the presence of heterogeneous patterns of various fungi (mostly saprophytic). It did not support the hypothesis of *Fusarium solani* being the cause of sissoo dieback. In contrast, isolation and molecular characterisation of bacteria from dieback affected sissoo revealed the presence of *Pseudomonas* in 83% of the samples. Sequencing the gene of 16S ribosomal RNA, the *rpoD*-gene, the *gacA*-gene and the *rnpB*-gene strongly suggested that these isolates belong most probably to a still unassigned *Pseudomonas* species. Hypersensitivity response assays and infection studies using sissoo seedlings demonstrated their pathogenic potential.

Introduction

Sissoo (*Dalbergia sissoo* Roxb.) is grown in tropical regions world-wide as a valuable timber. Its significant contribution to the socio-economic development of Bangladesh and the South Asian region is widely acknowledged (Webb and Hossain 2005). However, since 1993 the devastating dieback disease of sissoo has

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been recognized as a dramatic threat for forests and timber production in India, Nepal, Pakistan, Afghanistan and Bangladesh (Shukla 2002). The dieback syndrome is characterized by a combination of various disease symptoms. Wilting and subsequent loss of side branches, leading to so-called stagheads, as well as black gummosis on the trunk and discoloration of veins are considered very typical symptoms of dieback (Fig. 1A - D). Various fungi were reported to be found in dieback affected sissoo trees including *Fusarium solani* (Bakshi 1954, Manadhar and Shresta 2000) as well as *Cercospora sissoo*, *Phyllactinia dalbergiae*, *Phellinus dalbergiae* or *Ganoderma lucidum*, among many others (Dargan et al. 2002). However, since a broad diversity of fungi was found in samples from dieback affected sissoo and since the correlation of these fungi with dieback was not convincing (Baksha and Basak 2003), the causal agent(s) have not yet been unequivocally identified. Bacteria had been completely neglected in the past as putative agent of dieback, until authors could show through 16S rDNA sequence analyses that bacteria of the genus *Pseudomonas* were found in samples from heavily affected sissoo trees (Tantau et al. 2005). Diseases caused by *Pseudomonas* species including *P. syringae* and its pathovars are of major concern world-wide for forestry and cultivation of fruit trees (Kennelly et al. 2007). More recently, authors could show that viruses also may play a role in the complex etiology of sissoo dieback, because Vogel et al. (2011) found double stranded RNA and virus particles in leaf samples of dieback affected sissoo trees.

In order to elucidate the role of biotic agents in the dieback disease of sissoo, authors used a molecular approach to identify fungi and bacteria in leaf, twig, bark and root specimens from dieback affected *Dalbergia sissoo* trees, which had been collected from various sites in Bangladesh. Fungi were identified via sequence analyses of the ribosomal internal transcribed spacer (ITS) DNA, while bacteria of the genus *Pseudomonas* were detected and characterized via sequencing of taxon specific genes and the following questions were addressed: (a) association of a pathogenic fungal species with dieback, (b) the number of cases where *Pseudomonas* was found in dieback affected sissoo, (c) molecular characterization of these *Pseudomonas* isolates, and (d) pathogenic potential of *Pseudomonas* isolates.

Materials and Methods

Samples of leaves, twigs, barks and roots were collected from symptomless and from dieback affected *Dalbergia sissoo* Roxb. at various sites *viz.* Dhaka, Tangail, Sirajganj, Bogra, and along Jamuna Bridge Road, Rajshahi division, Bangladesh. Rating of severity of dieback symptoms (Table 1) was as follows: Class '0': means no typical symptoms of dieback, '1': mild symptoms such as leaf chlorosis and

necrosis and initial crown transparency, '2': medium symptoms such as strong leaf necrosis, advanced crown transparency and gummosis (black spots) at the bottom of the trunk, '3': severely affected trees with almost all foliage and branches lost (staghead) and many black spots on the whole trunk. Collected samples were kept cool in sealed plastic bags during transport and finally stored at -70°C .

For the detection of fungi, explants of sample materials (barks and roots) were surface disinfected and placed on three different agar media (Atlas and Snyder 2006, Pontecorvo et al. 1953 and Leach et al. 1982). Selected mycelia were subcultured until visual homogeneity and grown in submerge culture. DNA was extracted from the collected mycelia (Day and Shattock 1997) and analysed in 0.8% agarose gels. The following primer pairs were used for PCR and for sequencing in order to identify the isolated fungi: ITS4 and ITS5 (White et al. 1990) for the amplification of the ITS-region of various fungi, and the taxon selective ITS-fu primers (Abd Elsalam et al. 2003) for the detection of *Fusarium species*. Sequences of both strands of PCR products were obtained using Big Dye chain terminating mixture (Applied Biosystems, Darmstadt, Germany) as per the manufacturer's instructions, and used for database search (BLAST, NCBI).

Bacterial colonies were isolated by homogenising 1 g of surface disinfected sample material in 50 ml of sterile 0.8% NaCl and plating six ten-fold dilutions on King's B medium (King et al. 1954), GH medium and NS medium (Schaad 1980). Colony PCR and sequencing studies were done using the following primer pairs: 16S rDNA specific primers Y1 and Y2 (Young et al. 1991), and the taxon (*Pseudomonas*) selective primer pairs rpoD-f and rpoD-r (Mulet et al. 2009), gacA-1F and gacA2 (Costa et al. 2007) as well as rnpB-f (TAC GGA AAG TGC CAC AGA AAA) and rnpB-r (GGA GAG TCG ATC TRT AAG C) (Leif Kirsebom, personal communication).

Hypersensitivity response (HR) assays were performed by infiltration of bacteria, resuspended in sterile distilled water at a cell density of 5×10^7 or 1×10^8 per ml, into leaves of *Nicotiana tabacum* cv. Xanthi and *Chenopodium quinoa* as indicator plants. Plants were grown in the greenhouse under daylight conditions, temperature ranged from 16°C (night) to around 20°C (day).

Dalbergia sissoo seedlings used for infectivity assays were germinated from seeds (Sunshine-Seeds, Ahlen, Germany). According to the suppliers information the *D. sissoo* seeds were collected from 'normal' cultivars in tea garden plantations in North East India. Seedlings were cultivated in a greenhouse at around 20°C under daylight conditions supplemented with Poot HS 2000 (400W) lamps. Infectivity assays were performed either by infiltration of 10 leaves per seedling with bacteria (resuspended in sterile 0.8% NaCl), similar to the method

used for HR assay as described above, or secondly by injecting bacterial suspensions (approx. 200 to 500 μ l) into the axils at ten leaves per seedling, or thirdly by stem slashing by using razor blades dipped into a bacterial suspension before making cuts into the epidermis. Three months after inoculation seedlings were observed visually and bacteria colonies were isolated from homogenates of systemically infected leaves by using the technique described above. Bacterial colonies were tested by PCR using *gacA*-specific primers and the amplified DNA was sequenced.

For transmission electron microscopy (TEM) of bacterial isolates, cells from overnight cultures were observed with TEM Leo 906 E (Carl Zeiss, Oberkochen, Germany) after negative staining with uranyl acetate. Scanning electron microscopy (SEM) of plant material was done using the Philips XL-20 scanning EM. Specimen preparation followed standard procedures using critical point drying and gold coating with sputter coater SCD 030 (Bal-Tec, Liechtenstein).

Results and Discussion

Fungal mycelia were isolated from all dieback affected *Dalbergia sissoo* specimens (Table 1). DNA was extracted from all mycelia and subjected to PCR using two different primer pairs. PCR products obtained with the primer pair ITS4 and ITS5 were about 600 bp long (Fig. 2A). ITS4 and ITS5 primers had been proven to detect Ascomycetes, Basidiomycetes, Zygomycetes and Mastigomycetes (Larena et al. 1999). Sequence analyses revealed a broad spectrum of fungi associated with *D. sissoo* (Table 1). Fungi isolated from explants of sample P97, which was collected from outer bark of a symptomless, very old tree located at the Press Club, in the city of Dhaka, were identified as several species of *Trichoderma*. Species belonging to this genus are non pathogenic ubiquitous fungi, which are known antagonistic agents against pathogenic fungi. The detection of these species underlines the healthy status of control tree P97. From the second apparently healthy tree P91 no fungi could be detected at all (Table 1). *Bipolaris* species, *Curvularia affinis* and *Rhizopus microsporus*, all known as potentially pathogenic agents, were found on dieback affected sissoo trees showing mild symptoms (P5, P88). One species of the genus *Bipolaris*, *B. sorokiniana* is causing the spot blotch disease of wheat in South Asia (Jaiswal et al. 2007).

C. affinis is known as the causative agent of leaf spot disease of cereals (Huang and Zheng 2004), while *R. microsporus* harbouring endosymbiotic bacteria was described to be pathogenic for rice seedlings (Partinda-Martinez and Hertweck 2005). In samples from sissoo trees with medium dieback symptoms (P1, P83), again *R. microsporus* was detected together with *Mucor*

circinelloides, a ubiquitous non pathogenic soil fungus, *Trichoderma virens* and an unknown member of the genus *Fusarium*. With the exception of *M. circinelloides*, all other fungal species are known as disease agents of cereal plants, but any association with dieback diseases of trees has not been recognized before. From the severely affected sissoo trees P7 and P8 various fungi were detected. In P7 *Bipolaris* species was found, while only in P8 *Fusarium oxysporum* and in particular *Lasiodiplodia theobromae* could be clearly identified (Table 1). *F. oxysporum* was already discussed to be involved in sissoo dieback (Manandhar and Shresta 2000), whereas *Lasiodiplodia theobromae* is a well-known pathogen associated with dieback syndromes of various tropical trees, including *Mangifera indica* (Khanzada et al. 2004) and *Theobroma cacao* (Mbenoun et al. 2008). In all sissoo specimens with dieback, investigated in this study, the fungal pathogen *Fusarium solani*, which was claimed to be one of the major causes of sissoo dieback, was not detected by means of molecular characterisation through the ITS region. This finding was confirmed, when the taxon (*Fusarium*) selective ITS-fu primers were used (Fig. 2B). Although in two dieback affected trees (P1 and P8) *Fusarium* was detected by PCR, sequencing indicated *F. oxysporum* in P8 and an unknown species of *Fusarium* in P1 (Table 1).

Table 1. Detection of fungi in root and bark from dieback affected *Dalbergia sissoo* collected in Bangladesh through PCR using primer ITS4 and ITS5 and sequencing.

Sample	Site of collection	Symptom severity class	Fungal species	Sequence identity (%)
P91	Jamuna Bridge Road	0	no	
P97	Press Club, Dhaka	0	<i>Trichoderma atroviride</i>	99
			<i>T. koningiopsis/T. koningii</i>	99
			<i>T. ovalisporum</i>	99
P5	Tangail	1	<i>Bipolaris</i> species	97
			<i>Curvularia affinis</i>	98
P88	Jamuna Bridge Road	1	<i>Rhizopus microsporus</i>	95
P1	Tangail	2	<i>Rhizopus microsporus</i>	99
			<i>Mucor circinelloides</i>	99
			<i>Fusarium</i> species	99
P83	Jamuna Bridge Road	2	<i>Trichoderma virens</i>	99
P7	Tangail	3	<i>Bipolaris</i> species	94
P8	Tangail	3	<i>Lasiodiplodia theobromae</i>	99
			<i>Fusarium oxysporum</i>	99

Looking for bacteria as putative pathogens, authors had shown previously by sequence analyses of 16S rDNA that still unassigned bacteria belonging to the

genus *Pseudomonas* were associated with dieback affected sissoo trees (Tantau et al. 2005). While these samples were collected from sites around Sherpur, Bogra in the Rajshahi division, authors analysed in the meantime a broad collection of samples from 52 dieback affected and 15 unaffected *D. sissoo* trees at various sites

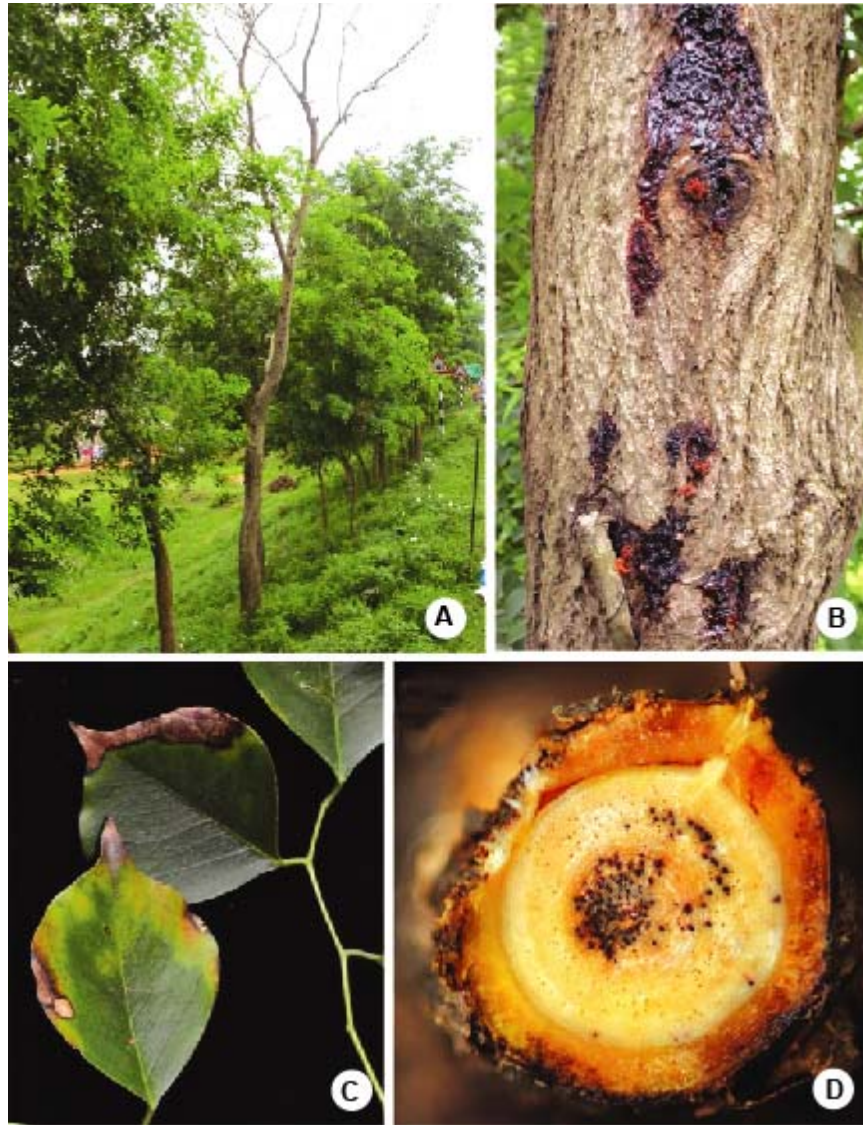


Fig. 1. *Dalbergia sissoo* showing symptoms of dieback. A. Severely affected tree with staghead. B. Black spots with gummosis on bark. C. Necrosis on leaves. D. Black discoloration of veins in root cut.

in and around Dhaka, Mirpur, Tangail, Sirajganj, Bogra and in plantations along Jamuna Bridge Road near Jamuna Bridge. When trees with typical symptoms of

dieback were investigated, bacteria of the genus *Pseudomonas* were detected in about 83% of 52 samples, while from apparently healthy trees without any dieback symptoms only in two of 15 samples (13.3%) *Pseudomonas* was found. The bacteria were detected in roots, bark, twigs and leaves of dieback affected *D. sissoo* trees. TEM inspection of selected isolates originating from dieback affected

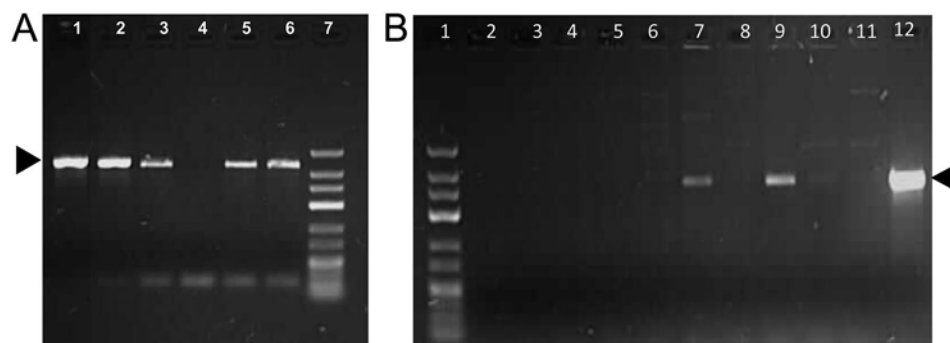


Fig. 2. Gel electrophoretic analysis of PCR products of fungal DNA from specimens of dieback affected *Dalbergia sissoo* trees in a 1.5% agarose gel. A. Primers ITS4 and ITS5. Lane-1. P1. Lane-2. P5. Lane-3. P8; Lane-4. Water, negative control. Lane-5. *Fusarium culmorum*. Lane-6. *Fusarium graminearum*; Lane-7. Gene Ruler DNA ladder low range (25 - 700 bp). Arrowhead indicates PCR products of approx. 550 - 600 bp in length. B. Taxon selective ITS-fu primers. Lane-1. Gene Ruler DNA ladder low range (25 - 700 bp); Lane-2. P88. Lane-3. P91. Lane-4. P97a. Lane-5. P97b. Lane-6. P1a. Lane-7. P1b. Lane-8. P1c. Lane-9. P8. Lane-10. P5. Lane-11. Water, negative control. Lane-12. *Fusarium culmorum*. Arrowhead indicates PCR products of approx. 450 bp in length. a, b and c indicate individual isolates from the same tree.

sissoo trees showed that the bacteria were about 1.4 to 2 μm in length and polar flagellated (Fig. 3). A total number of 63 isolates were detected and identified as *Pseudomonas* species by sequencing either the PCR fragment of the 16S rRNA gene (340 bp) or a PCR fragment of the *rpoD*-gene (RNA polymerase, 736 bp), or a fragment of the *rnpB*-gene for the RNA component of ribonuclease P (249 bp) and a 600 bp fragment of the single copy gene *gacA*, encoding a highly specific transcription regulator of *Pseudomonas*. Phylogenetic analyses by neighbour joining, based on sequences of PCR fragments of the 16S rRNA gene, the *rpoD*-gene, the *gacA*-gene and the *rnpB*-gene revealed that the isolated bacteria are closely related to some *Pseudomonas* species. This is shown exemplarily for a selection of few isolates (113, 1003, 1004, 1008 and 1160) in Fig. 4. When using the sequences of the rRNA gene (Fig. 4A), the *rpoD* sequences (Fig. 4B), the *gacA* sequences (Fig. 4C), and *rnpB* sequences (Fig. 4D), these isolates were grouped in two clusters close to plant pathogenic *Pseudomonas* species such as *P. fluorescens*, *P. oryzihabitans*, *P. viridiflava* and *P. syringae* as well as to some other species (*P. putida*, *P. fulva*, *P. stutzeri*, *P. oleovorans* and *P. aeruginosa*). Due to diverse sequence homologies of dieback associated *Pseudomonas* isolates to reference

strains of *Pseudomonas* species the isolates cannot be allocated to one of the known *Pseudomonas* species but rather represent strains of a yet unassigned species of *Pseudomonas*.

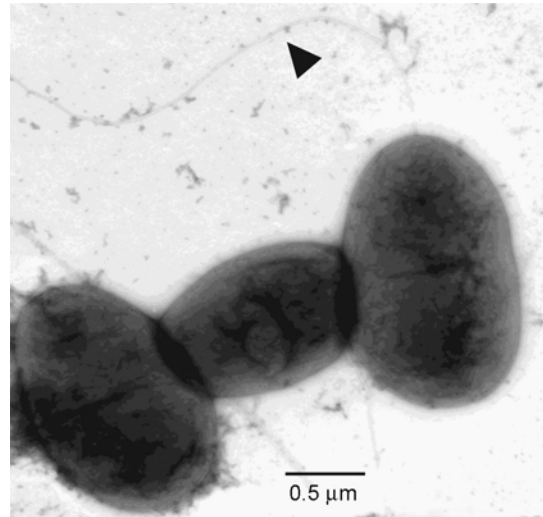


Fig. 3. Transmission electron microscopy of *Pseudomonas* isolate 1003 from dieback affected *Dalbergia sissoo*. Arrowhead indicates flagellum.

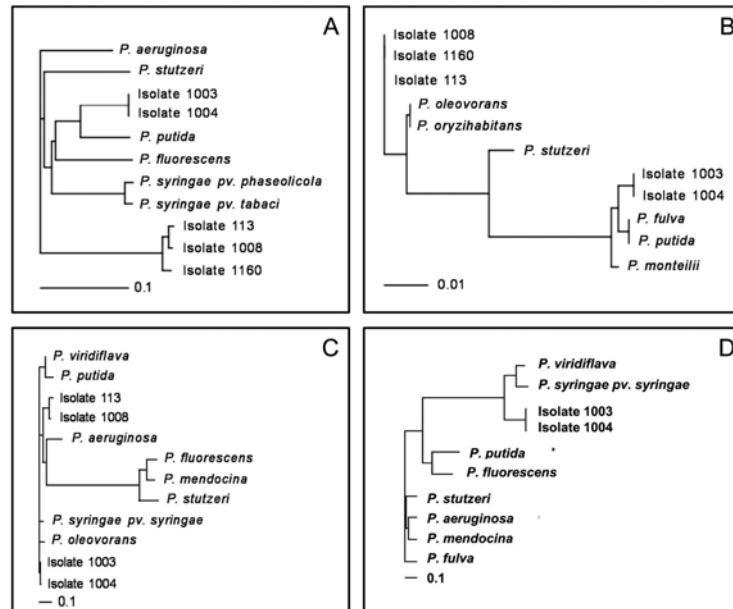


Fig. 4. Phylogenetic trees of sequences of PCR products of *Pseudomonas* isolates detected in dieback affected *Dalbergia sissoo*. A. *gacA*-gene sequences. B. 16S rDNA sequences. C. *rnpB*-gene sequences. D. *rpoD*-gene sequences. Trees were calculated by Neighbour Joining.

HR assays on indicator plants *Chenopodium quinoa* and *Nicotiana tabacum* cv. Xanthi revealed the pathogenic potential of selected bacterial isolates. A rapid hypersensitive response was obtained with most of our isolates after infiltration of bacterial suspensions into leaves of indicator plants which is exemplarily shown for three isolates (113, 1004 and 1008) in Fig. 5. These results strongly underline the phytopathogenic potential of such isolates.

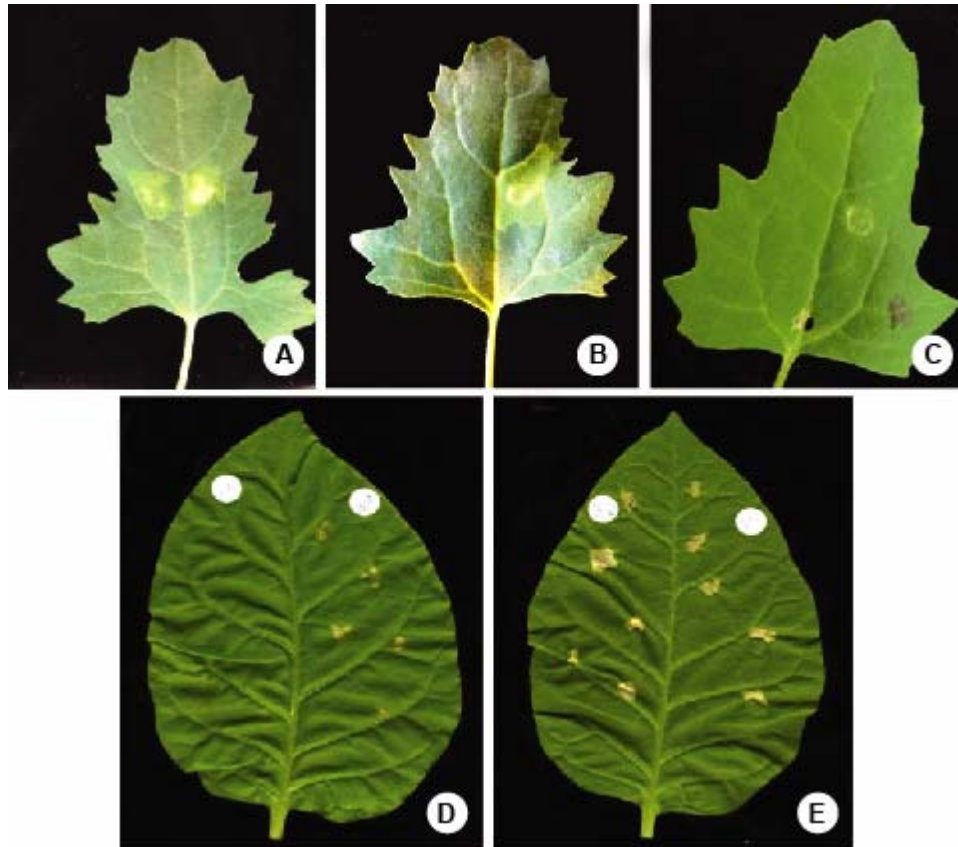


Fig. 5. Hypersensitivity Response (HR) assay on *Chenopodium quinoa* and *Nicotiana tabacum* cv. Xanthi of *Pseudomonas* isolates from dieback affected *Dalbergia sissoo*. A - C. *Chenopodium* leaves infiltrated with isolate 113 (A), isolate 1004 (B), and water (C). D. Tobacco leaf infiltrated with water as control (left half) and with isolate 1008 (right half). E. Tobacco (*N. tabacum* cv. Xanthi) leaf infiltrated with isolate 113. Cell density on left half was 5×10^7 cells per ml, on right half 1×10^8 cells per ml.

Experiments to infect *Dalbergia sissoo* seedlings by inoculation with *Pseudomonas* isolates under controlled conditions are currently under way. Authors used the isolates 113, 1003, 1004 and 1008, which among many others had been positively tested in HR assays (Valdez Aguirre 2010). Ten seedlings of

D. sissoo each were either inoculated by infiltration or by injection or by stem slashing. By visual inspection of the seedlings a strong response was found with isolate 1003, where after infiltration 8 of 10 seedlings showed necroses on leaves and stem (Table 2 and Fig. 6). Use of other isolates or inoculation of seedlings by injection and stem slashing led to less pronounced symptoms (Table 2). First results to re-isolate the inoculated bacteria from the treated seedlings after 3 months *p.i.* showed that the only means to infect *sissoo* seedlings was by infiltration of leaves. Using this technique the *Pseudomonas* isolates 1003 and 1004

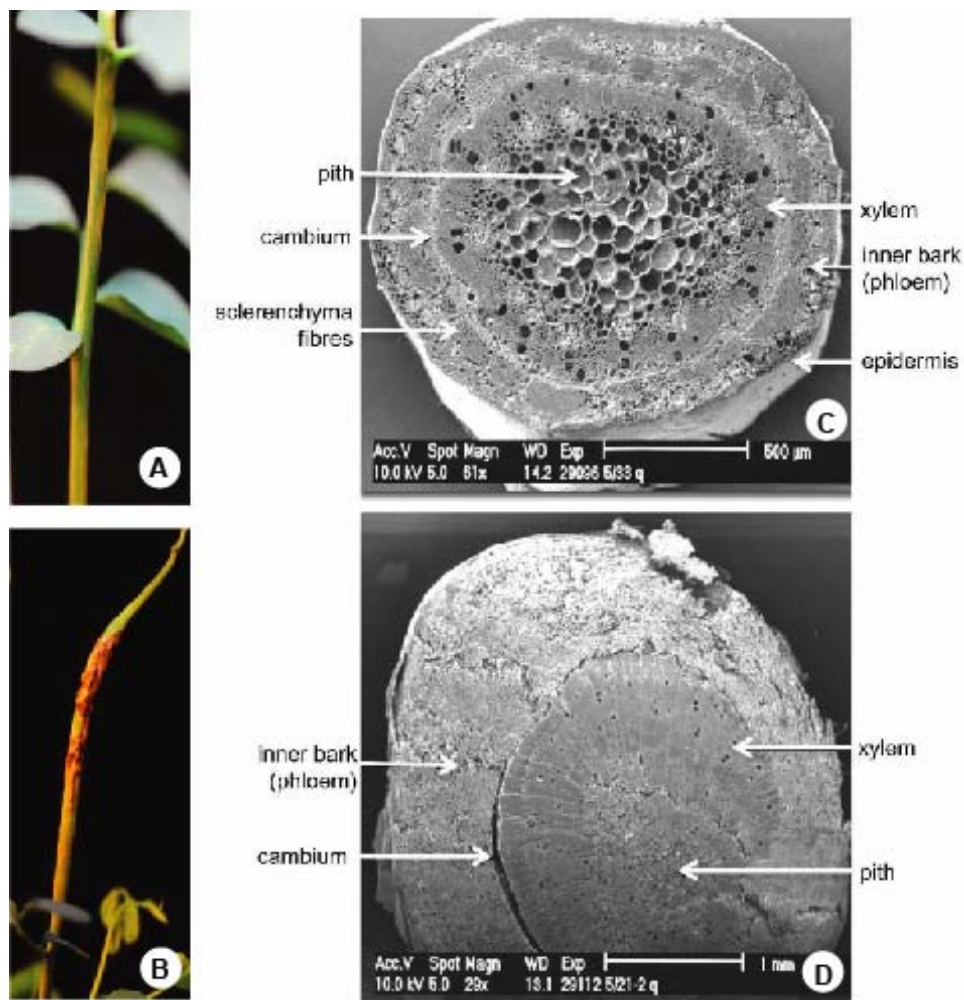


Fig. 6. Stem necroses on *Dalbergia sissoo* seedlings after infiltration with *Pseudomonas* isolate 1003. A. Control infiltrated with water. B. Seedling after infiltration with isolate 1003 with strong necroses on the stem. C. Scanning electron microscopy of a stem cut of control seedling. D. Scanning electron microscopy of a stem cut of a seedling infiltrated with isolate 1003. The cut was from the necrotic area shown in B.

could be re-isolated after 3 months from inoculated seedlings. After inoculation with *Pseudomonas* isolate 1003, 14 of 20 isolated colonies resulted in PCR products with the identical sequence as isolate 1003. Similar results were obtained with isolate 1004, where 2 of 8 inoculated seedlings showed necrotic symptoms after infiltration, from which eight colonies yielded *gacA*-positive PCR products with the identical sequence as isolate 1004 (Table 2). SEM studies of experimentally infected *D. sissoo* seedlings with strong necroses on the stem (Fig. 6B) revealed dramatic changes of the tissue (Fig. 6D). In cross sections the xylem veins and pith appeared completely destroyed and the surrounding tissue (inner bark and phloem veins) irregularly swollen, as compared to the control plants (Fig. 6C). It is reasonable to assume that these morphologic alterations strongly interfere with the function of the vascular tissue in water transport, which may lead to the typical dieback symptom of wilting in the initial stage of the disease.

Table 2. Response of *Dalbergia sissoo* seedlings to infiltration with *Pseudomonas* isolates after 3 months *p.i.* and re-isolation results of the inoculum.

Inoculum	Seedlings with necroses/inoculated seedlings	<i>gacA</i> -positive colonies	Sequence identity with inoculum
<i>Pseudomonas</i> isolate 113	4/8	0	
<i>Pseudomonas</i> isolate 1003	8/10	14	100% (all)
<i>Pseudomonas</i> isolate 1004	2/8	8	100 % (all)
<i>Pseudomonas</i> isolate 1008	4/8	0	
Control (H ₂ O)	0/10	0	

In conclusion, present findings provide clear evidence in favour of the hypothesis that the bacteria *Pseudomonas* play an important role in the etiology of the dieback disease of *Dalbergia sissoo*, while various pathogenic and opportunistic fungi may be involved as secondary parasites. However, intensive future efforts are required for understanding and possibly controlling of this disastrous disease.

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