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ROLE OF BACTERIA IN DIEBACK DISEASE OF *DALBERGIA SISSOO* ROXB.

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Abstract

The possible role of bacteria as pathogens in dieback affected *Dalbergia sissoo* Roxb. (sissoo) trees in Bangladesh has been investigated. Among the high diversity of bacterial genera detected by 16S rDNA sequences, several plant pathogenic *Pseudomonas* strains were identified and classified by AFLP analysis. Three isolates produced significant symptoms on *D. sissoo* seedlings after mechanical inoculation *in vivo*, suggesting that *Pseudomonas* should be considered as a potential cause for the dieback disease.

Introduction

Dalbergia sissoo Roxb. is next to teak (*Tectona grandis* L.f.) the most planted tree in South Asia (Orwa *et al*. 2009). Timber of *D. sissoo* finds multipurpose uses in different fields and has a high economical impact on many South Asian countries. Since the early 50s, this tree species has been affected by a disease called 'wilt of shisham', which was first detected in India and blamed to infection by *Fusarium solani* Snyder & Hansen (Bakshi 1954). While wilt of shisham was only observed in trees of about 15 - 25 years old (Bakshi 1954), a novel and probably different type of disease was observed in Bangladesh in the 1990s, which was also found in much younger trees in age classes from one up to 16 years (Basak *et al*. 2003). The disease has also been reported from Pakistan (Khan and Khan 2000) and Nepal (Sah *et al*. 2003). Various terms were used for the disease such as 'Mortality of sissoo' (Baksha and Basak 2000), 'Top dying disease' (Sah *et al*. 2003), 'Shisham decline' (Bajwa and Javaid 2007) and 'Dieback of sissoo' (Khan and Khan 2000), the latter one being used most frequently. The characteristic symptoms are yellowing and necrosis of the leaves, mostly starting from the top of the tree. Successive loss of leaves leads to the 'stag headed' symptom followed by occasional bark lesions with flow of dark red gum from the trunk.

Depending on the location, the progress of the disease varies (Dayaram *et al*. 2003), but in all cases the trees die within a few years. Drastic climatic changes such as the ones induced by the El Nino-Southern Oscillation (ENSO) could be a very important element in the spread of the disease (Collins *et al*. 2010). But rather than abiotic factors, various biotic agents have been discussed to be involved in the dieback disease of sissoo, such as the fungi *Fusarium solani* Snyder & Hansen, *Fusarium oxysporum* Schlecht *emend*. Snyder & Hansen, *Ganoderma lucidum* (Leyss.) Karst. and *Phellinus gilvus* Schwen. (Sharma *et al*. 2000, Baksha and Basak 2003). All of these organisms are known pathogens of *D. sissoo*, causing different types of symptoms. But since none of these organisms could be constantly isolated from dieback-affected trees, their role in the dieback of sissoo has not been convincingly demonstrated. Tantau *et al*. (2005) identified for the first time

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bacterial isolates of the genera *Bacillus* and *Pseudomonas* in affected trees, which initiated a program to search for biotic agents other than fungi in more detail. These studies also suggested that viral infection was associated with sissoo dieback (Vogel *et al*. 2011), but in particular the involvement of bacteria of the genus *Pseudomonas* could be further substantiated (Tantau *et al*. 2011). The aim of the present study was to investigate the potential role of bacteria in the dieback disease of the *D. sissoo*, addressing more general aspects. Therefore samples collected from several locations in Bangladesh were screened for the presence of plant pathogenic bacteria. Isolates with putative pathogenic potential were further analyzed on a molecular and physiological basis.

Materials and Methods

Leaves and roots of *Dalbergia sissoo* Roxb. plants were collected at four locations in Bangladesh from a total of 12 trees, exhibiting various stages of dieback disease, as shown in Table 1, and used for isolation of bacteria. Additionally, *D. sissoo* seedlings grown from seeds (Sunshine-Seeds, Ahlen, Germany) under greenhouse conditions were used for infiltration tests with isolated bacteria. In order to isolate bacteria, 1 g of leaves or roots from sampled trees was homogenized in 100 ml of a sterile 0.8% NaCl solution. The homogenate was diluted 1 : 100 and 1 : 10,000 in 0.8% NaCl. A volume of 50 and 500 µl of each dilution were plated on four different media: KM (King *et al*. 1954), LB (yeast reduced, Sambrook *et al*. 1989), YDC (Lakso *et al*. 1970) and NS (Schaad 1980), respectively. The plates were incubated at 28°C for two days. Selected bacterial colonies were streaked two more times on LB plates to obtain pure bacterial cultures.

The identification of the isolates was performed by single colony PCR using the universal primers Y1 and Y2 (Young *et al*. 1991). Each PCR-tube contained in a final volume of 25 µl the following reagents: 1× concentrated PCR buffer, 0.2 U Taq DNA polymerase, 0.2 mM dNTP-mix and 10 pmoles of each primer. The routine PCR program consisted of 30 cycles of 30 s denaturation at 94°C, 1 min annealing at 61°C and 90 s of elongation at 72°C. The PCR results were checked by electrophoresis in a 1.5% agarose gel. For sequencing 100 ng per 100 bp of PCR-product were mixed with 2 µl Big Dye Enzyme, 6 µl 2.5 × buffer (PE Biosystem) and 15 pmol Y1 or Y2 primer (25 cycles of 20 s denaturation at 96°C, 20 s annealing at 50°C and 4 min elongation at 60°C). Sequences were analyzed using the program DNAstar and search in the NCBI and EMBL databases. The sequences obtained in this work were deposited in EMBL, accession numbers are listed in Table 1.

Gram staining was used for the microbiological characterization of *Pseudomonas* isolates. The morphological appearance of the bacteria was recorded (colour and surface structure) on yeast reduced LB-medium (Sambrook *et al*. 1989) and standard microbiological enzymatic activity tests for catalase and oxidase were performed.

For the extraction of secondary metabolites, single colonies of *Pseudomonas* isolates were inoculated into 10 ml LB medium and cultivated under constant shaking (200 rpm, New Brunswick Innova 44) for 24 hrs at 28°C. Bacteria were removed by centrifugation (10 min at 4500 rpm) and the supernatant was extracted two times with one volume of ethyl acetate. The organic phase was concentrated to dryness in a vacuum evaporator and the remaining material was resuspended in 10 ml 0.8% NaCl solution.

The hypersensitivity response (HR) test for *Pseudomonas* isolates was performed using 4 ml aliquots of overnight cultures of bacteria. Cells were harvested by centrifugation, washed with 0.8% NaCl and resuspended in 300 µl of 0.8% NaCl. *Solanum lycopersicum* L., *Nicotiana tabacum* L. 'Xanthi' and *Chenopodium quinoa* Willd. were used as indicator plants. Usually, 100

µl of cell suspension were infiltrated into the intercostal area of the leaves (two leaves per plant and per isolate) using a 1 ml syringe without the needle. Necrosis or chlorosis at the infiltrated site obtained until the 4th day after inoculation was evaluated as a positive reaction. The extracted organic fractions were tested similarly but only on *N. t.* 'Xanthi'.

For amplified fragment length polymorphism (AFLP) genomic DNA was isolated from all *Pseudomonas* isolates by the CTAB method (Wilson 1997). The following reference strains were used: *Pseudomonas oleovorans* (DSMZ 1045), *P. corrugata* (DSMZ 7228), *P. syringae* (DSMZ 10604), *P. tremae* (DSMZ 16744), *P. savastanoi* (DSMZ 19341), *P. fluorescens* (DSMZ 50090), *P. cichorii* (DSMZ 50159), *Clavibacter michiganensis* (GSPB 3136). Extracted DNA was resuspended in TE-buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) to achieve a final concentration of 100 ng/µl. AFLP was performed according to Vos *et al*. (1995) modified by using Tru1I (Reineke and Karlovsky 2000) and fluorescent labeling. DNA (200 ng) was successively restricted with EcoRI and Tru1I in 10 µl reaction volume following the protocol given by Fermentas. After double digestion adapter molecules were ligated (EcoRI-Adapter 1: CTC GTA GAC TGC GTA CC, EcoRI-Adapter 2: AAT TGG TAC GCA GTC, Tru1I-Adapter 1: TAC TCA GGA CTC AT and Tru1I-Adapter 2: GAC GAT GAG TCC TGA G) with T4-DNA-ligase (Fermentas) in 20 µl reaction volume containing 10 µl double-restricted DNA, 5 pmol EcoRI-Adapter 1, 5 pmol EcoRI-Adapter 2, 50 pmol Tru1I-Adapter 1, 50 pmol Tru1I-Adapter 2, 1 × concentrated T4-DNA-ligase-buffer and 1 µl T4-DNA-Ligase. For preamplification 1 µl of a 1 : 10 dilution in TE-buffer of the restriction-ligation-solution was used as template in 25 µl PCR-reaction volume containing 10 pmol of EcoRI-00 primer (GAC TGC GTA CCA ATT C), 10 pmol Tru1I primer (GAC GAT GAG TCC TGA GTA A), 1× PCR-buffer (Bioline), 1.5 µl 50 mM $MgCl_2$ (Bioline), 0.5 µl 10 mM dNTP-mix (Fermentas) and 0.3 µl Taq-polymerase (Bioline). The preamplification PCR-reaction was performed in a Biometra thermocycler Tpersonal 48 by using the following protocol: 25 cycles of 30 s denaturation at 94°C, 1 min annealing at 56°C and 1 min elongation at 72°C. The preamplification products were then diluted 1 : 10 in TE-buffer and 1 µl was used as a template for the amplification with primers containing selective nucleotides. Each PCR reaction contained additionally 5 pmol labelled EcoRI primer, 10 pmol MseI primer, 1 × buffer (Bioline), 1.5 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP mix (Fermentas) and 1.5 u Taq-polymerase (Bioline). For this selective PCR six different primer combinations were used with following primers: EcoRI-A_Dy750 (GAC TGC GTA CCA ATT CA) and EcoRI-C_Dy681 (GAC TGC GTA CCA ATT CC) from Eurofins MWG Operon, MseI-A (GAC GAT GAG TCC TGA GTA AA), MseI-C (GAC GAT GAG TCC TGA GTA AC) and MseI-G (GAC GAT GAG TCC TGA GTA AG) from Invitrogen. The selective PCR was executed in a Biometra thermocycler Tpersonal 48 using the following protocol: 11 cycles of 30 s denaturation at 94°C, 30 s annealing starting at 65°C and 1 min elongation at 72°C. After each cycle the annealing temperature was decreased by 0.7°C. The first 11 cycles were followed by 30 cycles of 30 s denaturation at 94°C, 1 min annealing at 56°C and 1 min elongation at 72°C. Amplified fragments were analyzed by capillary electrophoresis using CQ8000 (Beckman Coulter, Brea, USA) under the following conditions: denaturation at 90°C for 120 s and separation for 80 min by 4.8 kV. The output of the Beckman Coulter Software is given as chromatograms, which were transformed into a presence/absence matrix. For fragment recognition the following parameter setting was used: a maximum Bin-Width (distance between the outer boundaries of each bin) of 1.25 nucleotides, a slope threshold of 10% and a relative peak height threshold of 10%. The confidence level was set as 95%. The applied model for calibration was the quartic curve model, which is recommended by Beckman to be selected when the Standard 600 of the company is being used. The matrix was then analyzed using the program NTSYS 2.0 (Rohlf 2000) for the clustering. The tree was calculated based on the similarity coefficient of Jaccard (1908) and clustered with the unweighted

combination group method using arithmetic averages (UPGMA). For bootstrap the program Winboot was used. Jaccard similarity was calculated on Winboot as well.

D. sissoo plants were inoculated with four different bacterial strains (isolates 113, 1003, 1004 and 1008) selected according to the results of AFLP analysis. 25 ml overnight cultures of each isolate were centrifuged at 3500 rpm for 10 min. The sediment was then suspended in 0.8% NaCl. The bacterial suspension (undiluted and diluted 1 : 10) was either injected into the axilla with a syringe (20G × 1½) or administered through infiltration into the intercostal areas of the leaves. Eighteen *D. sissoo* plants (between six and twelve months old) were inoculated with each isolate by infiltrating 20 leaves per plant and isolate and 10 axilla per plant and isolate, respectively. For each method, two plants were mock inoculated with 0.8% NaCl and water.

All statistical tests were performed using the software STATISTICA 8.0 (Statsoft, Tulsa, Oklahoma, USA). The symptom expression in *D. sissoo* leaves was assigned to levels (ranging from 1 = no symptoms to 4 = more than half of the plants with symptoms) and served as the dependent variable. Since the data did not meet the assumptions of normality it was analyzed with a generalized linear model (GLZ) using the logit link for ordinal data and model simplification with the factors 'treatment' (inoculation) and 'concentration' (pure or diluted 1 : 10) and their interaction. If a factor had a significant effect ($p < 0.05$) and consisted of more than two factor levels it was further analyzed using the non-parametric Kruskal-Wallis-ANOVA and a corresponding post hoc analysis for non-parametric data (Siegel and Castellan 1988). Since homoscedasticity is an assumption of the Kruskal-Wallis-ANOVA the equivalency of variance of the data was tested with the Brown and Forsythe-Test (Brown and Forsythe 1974) that is used to compare the variances of non-normally distributed data. The data for the control treatment of the infiltrated and injected *D. sissoo* leaves was pooled for statistical analysis since it was not significantly different according to the non-parametric Mann-Whitney-U-Test.

Results and Discussion

In the present investigation of the bacterial flora in *D. sissoo* trees, the genus of 118 isolates from leaves and 18 isolates from roots of dieback-affected and symptom-free trees was identified by microbiological testing, PCR of 16S rDNA and sequence analysis (Table 1).

Table 1. Bacteria isolated from *Dalbergia sissoo* trees showing different stages of dieback.

Geographical location of the sampled tree/tree identifier	Dieback symptoms[1]	Isolate	EMBL accession number	Putative genus determined by partial 16S rDNA sequence
Tangail / P5	No	1D	FN822930	*Pantoea*
Tangail / P5	No	1E	FN822931	*Curtobacterium*
Tangail / P5	No	1G	FN822932	*Pantoea*
Tangail / P5	No	1K	FN822933	*Pseudomonas*
Dhaka / 11-1	No	173	FN995135	*Klebsiella*
Dhaka / 11-1	No	175	FN995136	*Klebsiella*
Dhaka / 11-1	No	176	FN995137	*Pantoea*
Dhaka / 11-1	No	177	FN995138	*Pantoea*
Dhaka / 11-1	No	178	FN995139	*Pantoea*
Dhaka / 11-1	No	179	FN995140	*Enterobacter*

(Contd.)

Geographical location of the sampled tree/tree identifier	Dieback symptoms[1]	Isolate	EMBL accession number	Putative genus determined by partial 16S rDNA sequence
Dhaka / 11-1	No	180	FN995141	*Pantoea*
Dhaka / 11-1	No	181	FN995142	*Enterobacter*
Dhaka / 11-1	No	182	FN995143	*Pantoea*
Dhaka / 11-1	No	183	FN995144	*Aerococcus*
Dhaka / 12-1	No	161	FN995125	Uncultured bacterium
Dhaka / 12-1	No	163	FN995126	*Enterobacter*
Dhaka / 12-1	No	165	FN995127	*Klebsiella*
Dhaka / 12-1	No	166	FN995128	*Pantoea*
Dhaka / 12-1	No	167	FN995129	*Pantoea*
Dhaka / 12-1	No	168	FN995130	*Pantoea*
Dhaka / 12-1	No	169	FN995131	*Acinetobacter*
Dhaka / 12-1	No	170	FN995132	Uncultured bacterium
Dhaka / 12-1	No	171	FN995133	Uncultured bacterium
Dhaka / 12-1	No	172	FN995134	Uncultured bacterium
Jamuna Road / 8-1	Mild	194	FN995151	*Enterobacter*
Jamuna Road / 8-1	Mild	195	FN995152	*Staphyloccocus*
Jamuna Road / 8-1	Mild	196	FN995153	Uncultured bacterium
Jamuna Road / 8-1	Mild	198	FN995154	*Rhizobium*
Jamuna Road / 8-1	Mild	199	FN995155	*Enterobacter*
Jamuna Road / 8-1	Mild	1002	FN995156	Uncultured bacterium
Jamuna Road / 8-1	Mild	1003	FN995157	*Pseudomonas*
Jamuna Road / 8-1	Mild	1004	FN995158	*Pseudomonas*
Jamuna Road / 8-1	Mild	1006	FN995159	*Pantoea*
Jamuna Road / 8-1	Mild	1007	FN995160	*Pantoea*
Jamuna Road / 9-2	Mild	151	FN995116	*Staphylococcus*
Jamuna Road / 9-2	Mild	152	FN995117	*Pantoea*
Jamuna Road / 9-2	Mild	153	FN995118	*Pseudomonas*
Jamuna Road / 9-2	Mild	154	FN995119	*Pantoea*
Jamuna Road / 9-2	Mild	155	FN995120	*Pseudomonas*
Jamuna Road / 9-2	Mild	156	FN995121	*Enterococcus*
Jamuna Road / 9-2	Mild	157	FN995122	Uncultured bacterium
Jamuna Road / 9-2	Mild	158	FN995123	*Delftia*
Jamuna Road / 9-2	Mild	160	FN995124	*Sphingomonas*
Tangail / P1	Medium	115	FN822947	*Curtobacterium*
Tangail / P1	Medium	116	FN822948	*Curtobacterium*
Tangail / P1	Medium	117	FN822949	*Pseudomonas*
Tangail / P1	Medium	118	FN822950	*Curtobacterium*
Tangail / P1	Medium	119	FN822951	*Curtobacterium*
Tangail / P1	Medium	120	FN822952	*Curtobacterium*
Tangail / P1	Medium	121	FN822953	*Sphingomonas*
Tangail / P1	Medium	122	FN822954	*Sphingomonas*

(Contd.)

Geographical location of the sampled tree/tree identifier	Dieback symptoms[1]	Isolate	EMBL accession number	Putative genus determined by partial 16S rDNA sequence
Tangail / P1	Medium	123	FN822955	*Enterobacter*
Tangail / P1	Medium	124	FN822956	*Pantoea*
Tangail / P1	Medium	125	FN822957	*Acinetobacter*
Tangail / P6	Medium	126	FN822958	*Pseudomonas*
Tangail / P6	Medium	101	FN822934	*Pseudomonas*
Tangail / P6	Medium	102	FN822935	*Pseudomonas*
Tangail / P6	Medium	103	FN822936	*Pseudomonas*
Tangail / P6	Medium	104	FN822937	*Pantoea*
Tangail / P6	Medium	105	FN822938	*Pantoea*
Tangail / P6	Medium	106	FN822939	*Burkholderia*
Tangail / P6	Medium	107	FN822940	*Curtobacterium*
Tangail / P6	Medium	108	FN822941	*Pantoea*
Tangail / P6	Medium	109	FN822942	*Pantoea*
Tangail / P6	Medium	110	FN822943	*Pseudomonas*
Tangail / P6	Medium	112	FN822944	*Kocuria*
Tangail / P6	Medium	113	FN822945	*Pseudomonas*
Tangail / P6	Medium	114	FN822946	*Pseudomonas*
Tangail / P6	Medium	127	FN822959	*Pseudomonas*
Tangail / P6	Medium	140	FN822972	*Microbacterium*
Tangail / P6	Medium	141	FN822973	*Xanthomonas*
Jamuna Road / 6-2	Medium	143	FN822974	*Enterobacter*
Jamuna Road / 6-2	Medium	144	FN822975	*Sphingomonas*
Jamuna Road / 6-2	Medium	145	FN822976	*Pantoea*
Jamuna Road / 6-2	Medium	146	FN822977	*Pseudomonas*
Jamuna Road / 6-2	Medium	147	FN822978	*Pseudomonas*
Jamuna Road / 6-2	Medium	149	FN822979	*Pseudomonas*
Jamuna Road / 6-2	Medium	150	FN995115	*Pantoea*
Jamuna Road / 7-1	Medium	185	FN995145	Uncultured bacterium
Jamuna Road / 7-1	Medium	186	FN995146	*Rhizobium*
Jamuna Road / 7-1	Medium	187	FN995147	*Pantoea*
Jamuna Road / 7-1	Medium	190	FN995148	*Pantoea*
Jamuna Road / 7-1	Medium	191	FN995149	*Sphingomonas*
Jamuna Road / 7-1	Medium	192	FN995150	Uncultured bacterium
Bogra / 2-1	Medium	1032	FN995182	*Pseudomonas*
Bogra / 2-1	Medium	1033	FN995183	*Pseudomonas*
Bogra / 2-1	Medium	1034	FN995184	*Klebsiella*
Bogra / 2-1	Medium	1020	FN995173	*Klebsiella*
Bogra / 2-1	Medium	1021	FN995174	Uncultured bacterium
Bogra / 2-1	Medium	1023	FN995175	*Microbacterium*
Bogra / 2-1	Medium	1025	FN995176	Uncultured bacterium

(Contd.)

Geographical location of the sampled tree/tree identifier	Dieback symptoms[1]	Isolate	EMBL accession number	Putative genus determined by partial 16S rDNA sequence
Bogra / 2-1	Medium	1026	FN995177	Uncultured bacterium
Bogra / 2-1	Medium	1027	FN995178	*Pseudomonas*
Bogra / 2-1	Medium	1032	FN995182	*Pseudomonas*
Bogra / 2-1	Medium	1033	FN995183	*Pseudomonas*
Bogra / 2-1	Medium	1034	FN995184	*Klebsiella*
Tangail / P1	Medium	400	FN995185	*Pantoea*
Tangail / P1	Medium	401	FN995186	*Sphingomonas*
Tangail / P1	Medium	402	FN995187	*Pseudomonas*
Tangail / P1	Medium	403	FN995188	*Pseudomonas*
Tangail / P1	Medium	404	FN995189	*Pantoea*
Tangail / P1	Medium	405	FN995190	*Ralstonia*
Tangail / P1	Medium	406	FN995191	*Enterobacter*
Tangail / P1	Medium	408	FN995192	*Sphingomonas*
Tangail / P1	Medium	409	FN995193	*Acinetobacter*
Tangail / P1	Medium	410	FN995194	*Acinetobacter*
Tangail / P1	Medium	411	FN995195	*Klebsiella*
Tangail / P1	Medium	412	FN995196	*Rhizobium*
Tangail / P1	Medium	413	FN995197	Uncultured bacterium
Tangail / P1	Medium	414	FN995198	*Pseudomonas*
Tangail / P1	Medium	415	FN995199	*Enterobacter*
Tangail / P1	Medium	416	FN995200	*Pantoea*
Tangail / P1	Medium	418	FN995201	*Pantoea*
Tangail / P1	Medium	419	FN995202	*Klebsiella*
Tangail / P4	Severe	130	FN822962	*Pseudomonas*
Tangail / P4	Severe	131	FN822963	*Pseudomonas*
Tangail / P4	Severe	132	FN822964	*Enterobacter*
Tangail / P4	Severe	133	FN822965	*Agrobacterium*
Tangail / P4	Severe	134	FN822966	*Curtobacterium*
Tangail / P4	Severe	135	FN822967	*Rhizobium*
Tangail / P4	Severe	136	FN822968	*Bacillus*
Tangail / P4	Severe	137	FN822969	*Enterobacter*
Tangail / P4	Severe	138	FN822970	*Microbacterium*
Tangail / P4	Severe	139	FN822971	Uncultured bacterium
Bogra / 1-1	Severe	1008	FN995161	*Pseudomonas*
Bogra / 1-1	Severe	1009	FN995162	*Pantoea*
Bogra / 1-1	Severe	1010	FN995163	*Pantoea*
Bogra / 1-1	Severe	1011	FN995164	*Pseudomonas*
Bogra / 1-1	Severe	1012	FN995165	*Pantoea*
Bogra / 1-1	Severe	1013	FN995166	*Curtobacterium*
Bogra / 1-1	Severe	1014	FN995167	*Klebsiella*

(Contd.)

Geographical location of the sampled tree/tree identifier	Dieback Symptoms[1]	Isolate	EMBL accession number	Putative genus determined by partial 16S rDNA sequence
Bogra / 1-1	Severe	1015	FN995168	*Dyella*
Bogra / 1-1	Severe	1016	FN995169	Uncultured bacterium
Bogra / 1-1	Severe	1028	FN995179	Uncultured bacterium
Bogra / 1-1	Severe	1029	FN995180	*Pantoea*
Bogra / 1-1	Severe	1031	FN995181	*Pantoea*

[1]Dieback symptoms**:** No: Tree without typical dieback symptoms; mild: Chlorosis and necrosis on leaves as well as initial crown transparency; medium: Strong leaf necrosis, advanced crown transparency, gummosis and necrosis (black spots) at the bottom parts of the trunk; severe: Almost all foliage and most of twigs and branches of higher order lost (stagheadedness), black spots at the trunk up to at least 2 m height.

These 136 operational taxonomic units (OTUs) represented at least 19 different genera. Within this broad bacterial spectrum, the most prominent genera were *Pseudomonas* with 28 isolates and *Pantoea* with 14 isolates (Table 1). With one exception (isolate 1K) *Pseudomonas* isolates were only found in dieback-affected trees, predominantly at locations Tangail, Jamuna Road and Bogra, while *Pantoea* isolates were identified in all sampled trees in Bangladesh, irrespective of their disease status. Also, 11 isolates of the genus *Enterobacter,* known to comprise bacteria with plant pathogenic potential (Nishijima *et al*. 1987, Takahashi *et al*. 1997), and ten isolates of the Gram-positive and plant pathogenic genus *Curtobacterium* (Dunleavy 1989) were found in samples of dieback-affected as well as unaffected sissoo trees. A few γ-proteobacteria of the genus *Acinetobacter* and *Klebsiella*, not yet known as plant pathogenic agents, were additionally found in unaffected and dieback-affected trees. Six isolates of the genus *Sphingomonas* were identified in dieback-affected trees only, as was the case for the sporadically occurring isolates of other plant pathogenic Gram-negative genera, such as *Agrobacterium* (Escobar and Dandekar 2003), *Burkholderia* (Coenye and Vandamme 2003), *Xanthomonas* (Kay and Bonas 2009, White and Yang 2009), *Ralstonia* (Guidot *et al*. 2009) and *Rhizobium*. Rarely further Gram-positive bacteria were found such as *Kocuria*, *Microbacterium*, and *Staphylococcus*. All these isolates were associated with dieback-affected sissoo samples. With the obtained 16S rDNA sequences a phylogram was constructed (Fig. 1) that showed two clusters of *Pseudomonas* (marked blue) and of *Pantoea* (marked orange), suggesting the presence of more than one species in both cases.

The genus *Pantoea* comprises eight species that are generally related with plants either as pathogen or as epi-/endophyte (Brady *et al.* 2011, Delétoile *et al*. 2009). Present authors found the *Pantoea* isolates distributed in two clusters with very high bootstrap values of 100 and 98%, respectively (Fig. 1), suggesting at least two different species of *Pantoea*. Seven other isolates grouping close to the *Pantoea* cluster could not be identified, but the bootstrap values showed that these must be either very closely related bacteria (i.e. genus *Enterobacter*, isolate 179) or a *Pantoea* species that has not been characterized so far (1002, 1021). One *Pantoea*-cluster (orange marked isolates with a bootstrap value of 100%) is built up only by isolates from affected trees. In the other *Pantoea*-cluster various isolates were also found in two trees without dieback-symptoms (Table 1).

The cluster of *Pseudomonas* isolates is also distributed into two subclusters, a highly homogeneous one, where the majority of the isolates are found, while a minority of only six isolates was clustering in a more heterogeneous subcluster. This cluster organization probably reflects the presence of at least two species of this highly complex genus among the bacteria

Colour

Fig. 1. Phylogram of the bacterial isolates from *Dalbergia sissoo*, based on 16S rDNA sequences. Clusters framed blue show *Pseudomonas* isolates, clusters framed orange show *Pantoea* isolates. The phylogram was constructed using program MEGA4, grouping of isolates was based on Neighbour Joining.

isolated in this study. The almost exclusive association of the well-known plant pathogenic genus *Pseudomonas* (Kennelly *et al*. 2007) with dieback affected sissoo trees prompted to investigate these isolates in more detail. Also, bacteria of this genus had been identified in earlier studies on sissoo dieback (Tantau *et al*. 2005). Biochemical analyzes showed that, as expected, all isolates identified as *Pseudomonas* by 16S rDNA sequencing were Gram-negative and catalase positive. However, the isolates differed in their oxidase activity. Only the isolates 103, 126, 146, 147, 149, 153, 402, 403 and 1011 showed oxidase activity, while all other isolates did not (Fig. 2B).

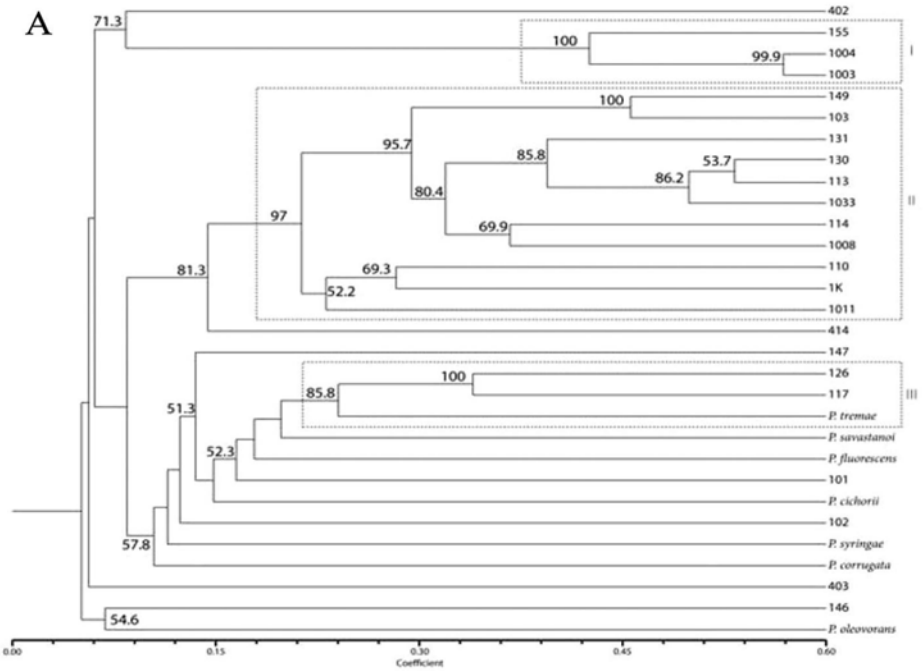
In addition to 16S rDNA sequences, 23 of the *Pseudomonas* isolates were further analyzed by AFLP. The analysis revealed three main clusters, clearly separated from each other by high bootstrap values (Fig. 2A, clusters I, II and III with the respective bootstrap values of 100, 97 and 85.8% each). From the reference strains of *Pseudomonas* used in this study, only *Pseudomonas tremae* grouped with a bootstrap value of 85.8% to cluster III, none of the others grouped within any of the clusters. Interestingly, all isolates found in clusters II and III are clustering together in a very homogeneous subcluster (bootstrap value 100%) based on 16S rRNA sequences (Fig. 1), while the isolates of cluster I (155, 1003 and 1004) are represented in the minor *Pseudomonas* subcluster (Fig. 1).

To limit the characterization of isolates to those potentially involved in the dieback disease of sissoo, their ability to evoke a hypersensitive response (HR) on indicator plants was tested. An isolate was considered pathogenic if it induced a positive HR in at least one of the three indicator plant species. Some *Pseudomonas* isolates provoked an untypical phenotype on the infiltrated leaves of *S. lycopersicum*, which appeared as red pigmentation all over the infiltrated leaf surface. With only two exceptions (isolates 147 and 1011) all the other tested 21 *Pseudomonas* isolates were able to induce a hypersensitive response in at least one of the indicator plants, confirming their plant pathogenic potential (Fig. 2B). There was an interesting coincidence between the ability to induce HR on indicator plants and the clusters built on AFLP basis (Fig. 2). Even the untypical reaction on tomato plants could be observed in all three isolates of cluster I as well as in the isolates 110, 1K, 1011, which build a subcluster in cluster II. The enzyme activity of oxidase is also reflected in the clustering. For instance, isolates 149 and 103 share the same features and group together in a subcluster of cluster II by a bootstrap value of 100%. Cluster I is very uniform with respect to the results of all investigations. The three isolates stem from trees at the nearby located sites Tangail and Jamuna Road, proved to be HR-positive in *C. quinoa* and *N. t.* 'Xanthi' showing an untypical HR in tomato plants and a lack of oxidase activity.

In contrast, cluster II harbors isolates from two regions (Tangail and Bogra), which behave differently in the HR assay and show different morphological characteristics. Except for the isolates 1K, 110, 113 and 1011, all isolates in this cluster were clearly identified as HR-positive in all tested plants (including *S. lycopersicum*). OTUs in cluster II that were building subclusters showed the same biological and biochemical characteristics in most of the cases, for example the isolates 149 and 103 or 114 and 1008. Isolates 117 and 126, which together with the plant pathogenic reference strain *P. tremae* build cluster III, originate from two trees located at Tangail. These isolates were tested HR-positive in all plants, but differed in oxidase activity. All *Pseudomonas* isolates from roots (402, 414 and 403), which were found in samples from a tree at Tangail with medium dieback-symptoms, are distantly related according to their individual position in the AFLP dendrogram. Although, all proved positive in HR assays and 402 and 403 showed oxidase activity (Fig. 2A and B). Among the isolates that are outside of these three main clusters no significant correspondence of their characteristics was found.

Bacteria can harm their hosts in various ways for example through the secretion of enzymes (Magro *et al*. 1994), polysaccharides (Denny 1995), phytohormones (Abramovich *et al*. 2006, Yang *et al*. 2007) or secondary metabolites. Among the secondary metabolites, the toxins are of

A



B

	Isolate	Location	HR Quinoa	HR Xanthi	HR Tomato	HR Ethylacetate extract	Oxidase
	402	Tangail	-	-	+	-	+
	155	Tangail	+	+	UT	-/+	-
I	1004	Jamuna Road	+	+	UT	+	-
	1003	Jamuna Road	+	+	UT	+	-
	149	Tangail	+	+	+	-	(+)
	103	Tangail	+	+	+	-	(+)
	131	Tangail	+	+	+	-	-
	130	Tangail	+	+	+	-	-
II	113	Tangail	-	+	+	-	-
	1033	Bogra	+	+	+	-	-
	114	Tangail	+	+	+	-	-
	1008	Bogra	+	+	+	-	-
	110	Tangail	-	+	UT	-	-
	1K	Tangail	+	-	UT	-	-
	1011	Bogra	-	-	UT	-	(+)
	414	Tangail	-	+	UT	-	-
	147	Tangail	-	-	UT	-	+
	126	Tangail	+	+	+	-	(+)
III	117	Tangail	+	+	+	-	-
	101	Tangail	+	-	UT	-	-
	102	Tangail	+	+	+	-	-
	403	Tangail	-	+	+	-	+
	146	Tangail	-	+	+	-	(+)

Fig. 2. A: Dendrogram of the AFLP analysis of *Pseudomonas* isolates. The dendrogram was calculated with Jaccard similarities and using UPGMA for clustering. All significant bootstrap values are specified on the respective node. The cophenetic correlation coefficient is r = 0.95661. The clusters to be considered in this analysis are framed (I, II, III). B: Hypersensitive response assays (HR) and oxidase activity assays of the respective *Pseudomonas* isolates on indicator plants. HR Quinoa: *Chenopodium quinoa*; HR 'Xanthi': *Nicotiana tabacum cv.* 'Xanthi'; HR tomato: *Solanum lycopersicum*. HR ethylacetate extract: Extract tested on *N. t.* 'Xanthi'. +: positive reaction, (+): Retarded positive reaction, –: Negative reaction; UT: Untypical symptoms**.**

great importance for plant pathology (Amusa 2006). In the case of *Pseudomonas*, there are several species producing toxins: *P. syringae* produces coronatine, tabtoxin and phaseolotoxin, *P. corrugata* produces corpeptin and *P. marginalis* produces viscosin (Bender *et al*. 1999, Kimura *et al.* 2001). In order to investigate whether the pathogenic potential of *Pseudomonas* isolates is based on toxic compounds, the effect of extracted secondary metabolites was tested. Among all tested isolates only the extracts of isolates 1003 and 1004 induced typical necrotic lesions on tobacco leaves. Interestingly, the secondary metabolite extract of isolate 155, which groups close to 1003 and 1004 both in 16S rRNA and AFLP analyses, also induced necrotic lesions in the HR assay, though less remarkable ones.

Among the broad spectrum of bacterial genera isolated from *D. sissoo* trees the dominant presence of members of the genus *Pseudomonas* is noticeable. This genus includes plant pathogenic as well as saprophytic members, which might be present as opportunistic agents instead of being the cause of the disease itself. Therefore, their ability to provoke disease symptoms in sissoo seedlings after mechanical administration of selected isolates was investigated.

Colour

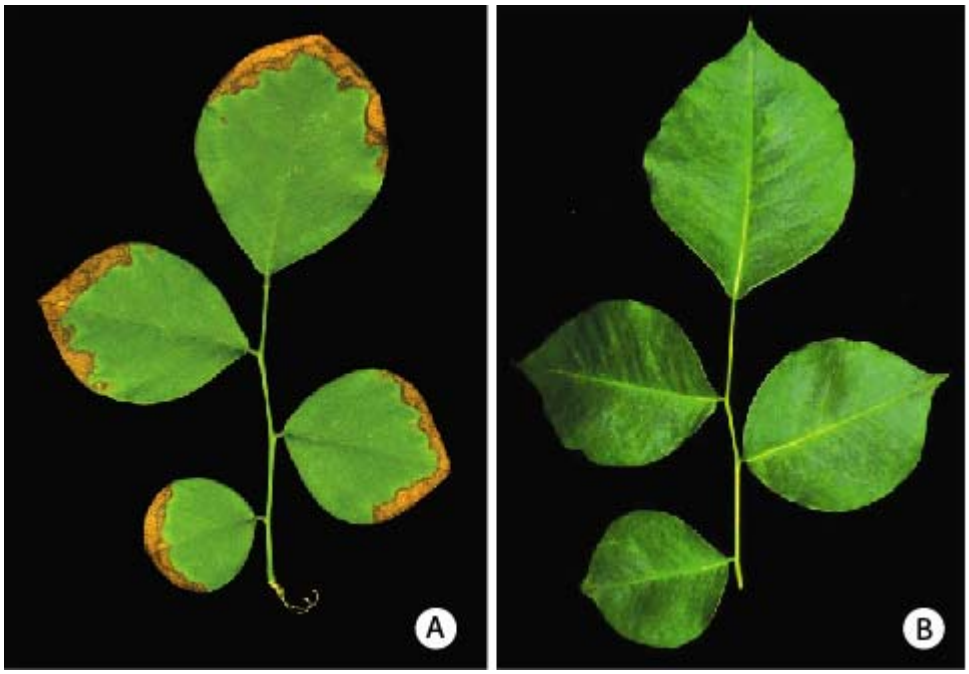


Fig. 3A-B**.** A. Typical disease symptoms (necroses) on the leaf of a *Dalbergia sissoo* seedling after infiltration with *Pseudomonas* bacteria isolated from a dieback affected sissoo tree in Bangladesh. B. Leaf from a sissoo seedling infiltrated with water.

Representative isolates of cluster I (1003 and 1004) and cluster II (113 and 1008) shown in Fig. 2 were used for inoculation experiments on sissoo seedlings. Isolates 1003 and 1004 were both sampled from the same tree at Jamuna Road and proved HR positive in *C. quinoa* and *N. t.* 'Xanthi'. Isolate 113 was sampled from a diseased tree at Tangail and was tested HR positive in all plants but *C. quinoa*. Isolate 1008 originated from a diseased tree at Bogra and was invariably HR positive among all indicator plants.

When inoculated with the *Pseudomonas* isolates 113, 1003 and 1008, *D. sissoo* trees showed strong necrosis on the inoculated leaves, while inoculation with isolate 1004 caused fewer and weaker symptoms. The data were recorded over a period of 5 - 13 months post inoculation. In general, necroses were predominantly found at the leaf margin (Fig. 3A) which is in contrast to necrotic spots observed on leaves of 12 - 15 years old dieback affected trees in Bangladesh. Those lesions were more frequently distributed on the lamina. Leaves of control plants, treated with water, did not show any necrotic alterations (Fig. 3B). The statistical analysis revealed a significant effect of the treatment with bacteria at all, (GLZ, df = 3, p = 0.036) but not of the mass of bacteria administered (GLZ, df = 1, p = 0.998, data not shown). With the exception of the isolate 1004, the other three isolates caused symptoms in *D. sissoo* which significantly differed from the control treatment (Table 2).

Table 2. Statistical analysis of the symptom expression in *Dalbergia sissoo* plants after inoculation with bacteria isolated from dieback-affected sissoo trees.

Treatment	Symptom expression (Mean ± SE)	Number of replicates	P-value
Control	1.76 ± 0.25	4	-
Isolate 113	3.75 ± 0.15	8	0.0232
Isolate 1003	3.6 ± 0.31	10	0.0203
Isolate 1004	2.5 ± 0.19	8	1
Isolate 1008	4 ± 0	10	0.0025

Data were analyzed using the Kruskal-Wallis-ANOVA and a corresponding post hoc analysis for non-parametric data. All isolates were tested against the control treatment.

Although infection experiments on trees are known to be very difficult to perform, a clear response of the natural host species after inoculation with three of the here isolated *Pseudomonas* strains was observed. The importance of the genus among tree diseases has been reported before (Cazorla *et al.* 1998, Menard *et al.* 2003, Scortichini *et al*. 2005, Green *et al.* 2010) and the efforts to understand the pathogenicity of *Pseudomonas* have provided new approaches for the detection and isolation of this pathogen. There is growing evidence for some *Pseudomonas* species to have the ability to modify host metabolism to their own advantage through effectors and toxins (Rico *et al.* 2011). Such metabolic modifications might explain some of the typical dieback symptoms of sissoo trees like necrosis and successive loss of the crown as a consequence of disturbed water transport. Furthermore, some *Pseudomonas* toxins and effectors are known to interfere with chloroplast functions, which results in nitrogen mobilization and suppression of plant defense, enabling the bacteria to proliferate inside the plant (Rico *et al*. 2011). In addition to toxins and effectors many *Pseudomonas syringae* strains produce plant hormones or plant hormone inducers (e.g. indole-3-acetic acid, γ-aminobutyric acid) manipulating and affecting the plant physiology (Cao *et al.* 2011). Interestingly, a recent scanning electron microscopic investigation of *D. sissoo* seedlings, which were experimentally infected with *Pseudomonas* isolate 1003, revealed cancerous proliferation of phloem tissue and stem necrosis (Tantau *et al*. 2011). The impact of the bacterial intervention in this way is still not well understood, though it is certainly an important key to the understanding of the infection process of many plant diseases.

Identifying bacteria from the genus *Pseudomonas* is a very difficult task even when using the most modern techniques available (Ait Tayeb *et al.* 2005). Identifying a specific bacterium as the potential cause of a disease is even more so. Nevertheless, in the present investigation not only the association of *Pseudomonas* with dieback-affected *D. sissoo* trees was confirmed, but also the pathogenic potential of *Pseudomonas* isolates could be demonstrated.

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