Pak. J. Bot., 46(4): 1431-1435, 2014.

ISOLATION AND 16S rDNA SEQUENCE ANALYSIS OF BACTERIA FROM DIEBACK AFFECTED MANGO ORCHARDS IN SOUTHERN PAKISTAN

ISHTIAQ A. KHAN[1], ASIFULLAH KHAN[1], HUMA ASIF[1], M. MITHAL JISKANI[3], HANS-PETER MÜHLBACH[2] AND M. KAMRAN AZIM[1*]

[1]H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan
[2]Biocenter Klein Flottbek and Botanical Garden, University of Hamburg, Ohnhorststr. 18, 22609, Hamburg, Germany
[3]Department of Plant Pathology, Sindh Agriculture University, Tandojam, Pakistan
**Corresponding author e-mail: kamran.azim@iccs.edu; Tel.; +9221-111222292 ext. 236*

Abstract

A broad range of microorganisms are involved in various mango plant diseases such as fungi, algae and bacteria. In order to study the role of bacteria in mango dieback, a survey of infected mango plants in southern Pakistan was carried out. A number of bacterial isolates were obtained from healthy looking and infected mango trees, and their characterization was undertaken by colony PCR and subsequent sequence analysis of 16S rDNA. These analyses revealed the presence of various genera including *Acinetobacter, Bacillus, Burkholderia, Cronobacter, Curtobacterium, Enterobacter, Erwinia, Exiguobacterium, Halotelea, Lysinibacillus, Micrococcus, Microbacterium, Pantoea, Pseudomonas, Salmonella* and *Staphylococcus*. It is noteworthy that several members of these genera have been reported as plant pathogens. The present study provided baseline information regarding the phytopathogenic bacteria associated with mango trees in southern Pakistan.

Introduction

Mango (*Mangifera indica* L.) is an important fruit crop of the tropical and subtropical countries (Litz, 2009). The mango tree is considered to have evolved in the rainforests of South and South-east Asia (Knight, 1980; Krishna & Singh, 2007). Mango is commercially grown in over hundred countries. Major mango growing countries are India, China, Pakistan, Indonesia, Thailand, Philipines, Australia, Nigeria, Myanmar, Egypt and Bangladesh. Full-grown *Mangifera indica* trees reach a height of 40 meters and can stay alive for several hundred years.

Mango trees suffer from many devastating diseases (Lim & Khoo, 1985; Iqbal *et al*., 2006; Rajput & Rao, 2007). A range of microorganisms are involved in these diseases such as fungi, algae and bacteria (Litz, 2009). These microbes cause sets of symptoms including dieback, spots, necrosis, mildew scab, blotch, anthracnose and rots in mango trees (Ploetz, 2003; Freeman *et al*., 1999; Haggag & Abd El-Wahab, 2009). *Pseudomonas syringae* and *Xanthomonas* species (causing 'apical necrosis' and 'bacterial black spot' respectively) are among the few known bacterial pathogens of mango trees (Cazorla *et al*., 1998; Pruvost *et al*., 2005; Ah-You *et al*., 2007). Strains of *P. syringae* produce a secondary metabolite, mangotoxin which is involved in pathogenicity (Arrebola *et al*., 2007; Mraz *et al*., 1999). Bacterial black spot of mango trees has been reported from several mango producing countries i.e. India, Kenya, Pakistan, Malaysia, Comoros, Australia, Myanmar and Philippines.

Currently, mango trees in southern Pakistan are suffering from a disease with symptoms like "dieback disease". The characteristic symptoms are yellow necrotic leaves, lesions on the trunk bark and oozing of brownish sap from the stem. The detailed characterization this mango dieback disease and the causative agent(s) are required. Plant infections caused by bacterial pathogens are for the most part disapproving due to non-availability of post-infection remedies. The long time between introduction of bacteria and detection of the infection (i.e. from days to years) makes successful control of disease very complicated (Schaad *et al*., 2003). Therefore, screening of these plant pathogens are required to start agronomic measures or carry on suppression actions to evade the overwhelming outcomes.

In the present study, we characterized bacterial species associated with mango trees using bacteriological and molecular biology procedures. Bacteria from healthy and infected leaves and bark samples from mango trees cultivated in Karachi and Tando Jam regions in southern Pakistan were cultured, isolated and characterized by colony PCR and DNA sequencing.

Material and Methods

The samples were collected from Karachi and Tando Jam districts of Pakistan. Four samples were collected from diseased plants, two from Karachi (sample codes: MH-1 and MI-1) and two from Tando Jam (sample codes: TJI-1 and TJI-3). The sample MH-1 was collected from a healthy plant in Karachi. Samples from Tando Jam were bark samples while those collected from Karachi were leaves. The surface of plant material was sterilized with 1.3% sodium hypochlorite for 10 minutes and rinsed thrice with sterile water. The perimeters of samples were removed using a sterilized cutter and the sample was cut into tiny pieces.

Hundred milligrams of sample material was placed in to a mortar containing small amount of sea sand and 5 ml of sterilized 0.8% sodium chloride solution. This mixture was then homogenized thoroughly with a pestle and kept at 4°C. Aliquots of undiluted and diluted homogenates (100 µl) were inoculated on LB medium plates and incubated at 37°C for 24-48 hours. The bacterial colonies appeared on 'master-plates' were sub-cultured on new LB agar plates repetitively until a uniform population of separated colonies was achieved. Gram staining was performed to differentiate as gram positive or gram negative bacterial colonies.

Bacterial colonies were directly subjected to PCR amplification according to Hartung *et al*., (1998). The forward primer Y1 (5´-GGCTCAGAACG AACGCT GGCGGC-3´) and the reverse primer Y2 (5´-CCCACT GCTGCCTCCCGTAGGAGT-3´) (Young *et al*., 1991) was used. These primers are highly specific for conserved regions of bacterial 16S ribosomal DNA. The PCR reaction mixture (50 µl) was prepared by adding 1 Unit *Taq* DNA polymerase, 5 µl 10x PCR buffer (Roche Diagnostics GmbH, Mannheim, Germany), 0.2 mM dNTPs and 0.5 µM of each primer. An undetermined number of cells were picked from bacterial colonies on culture plates and immediately transferred into PCR tubes (Hartung *et al*., 1998). PCR was carried out with program as follows; an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 61 °C for 60 seconds, 72°C for 90 sseconds, 94°C for 30 seconds, with a final annealing step of 61°C for 2 minutes and a final extension step of 72°C for 5 minuntes. The PCR products were analyzed by agarose gel electrophoresis (1%) and detected by EtBr staining.

The purified PCR products were sequenced using Y1/Y2 primers and the Big Dye Terminator Kit (Applied Biosystems Inc., USA). DNA sequencing was carried out by Genetic Analyzer ABI 377 (Applied Biosystems Inc., USA). The 16S rDNA sequences were analyzed with BLAST (Altschul *et al*., 1990) using Staden Program (Staden, 1996) and DNASTAR-Lasergene sequence analysis package (DNASTAR Inc., Madison, USA).

Results and Discussion

Mango is suffering from a devastating disease due to which plant eventually dies. Symptoms of this disease are like “dieback disease” of *Dalbergia sissoo* (Sissoo, Tali or shisham) but this inference still has a shade of ambiguity as a comprehensive study on this disorder has not been conducted. The initial distinguishing symptoms of sissoo dieback infection are yellow necrotic leaves. Afterwards, thinning and wilting of leaves and crown also occur. With progressive development of infection, the affected trees show minute, longitudinal and exudates producing scratches on the trunk bark. Brownish sap oozes out, solidifies on the bark and afterward turns blackish. This coloration may extend from roots up to 3–4 meters on the stem (Baksha & Basak, 2003, Tantau *et al*., 2005). Similar symptoms were also observed in the sample trees under study (Fig. 1).

A total of four samples from leaf and bark of mango trees were processed in this study. About ninety isolated bacterial colonies were obtained. Microbiological and PCR characterization of 56 colonies were performed. Colony PCR of 33 colonies resulted in good amplified products, while 16 yielded only light bands on agarose gels (Fig. 2). From seven colonies, PCR product could not be obtained. Forty PCR products were subjected to DNA sequencing using same 16S rDNA primers. Finally 16S rDNA sequences of 34 bacterial samples were obtained. As a result of BLAST searches of the obtained sequence data, different types of soil and plant associated bacteria genera were identified, few of which are established plant pathogens. The details of these results are given in Tables 1 and 2.

Fig. 1. Disease symptoms on dieback affected mango trees in (a) Karachi and (b) Tando Jam, both in Sindh province of Pakistan.

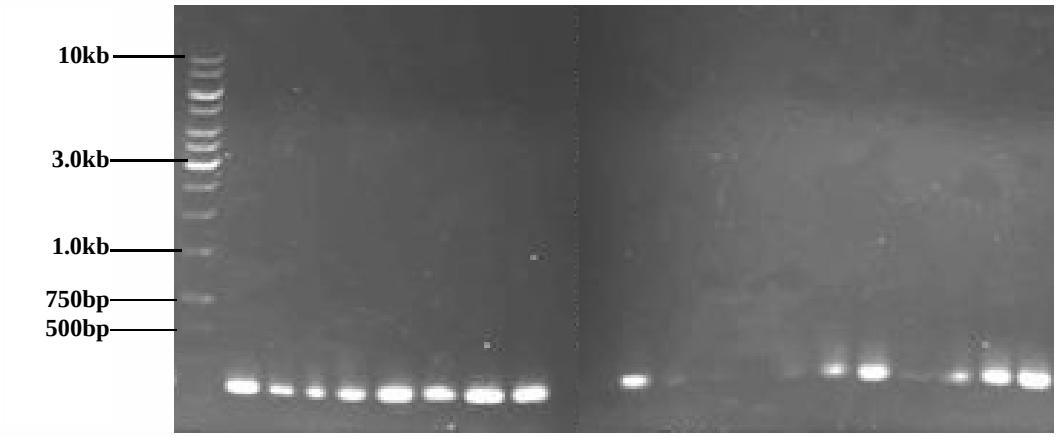

Fig. 2. Electrophoresis of colony PCR products from bacterial colonies isolated from mango leavers/barks.

Table 1. Isolate IDs, genbank accession numbers and blast identification of bacteria isolated from mango trees from Karachi, Pakistan.

No.	Isolate ID	Sample ID	Genbank accession	Colony PCR	Blast identification
1.	MH-1	MH-I	JQ266319	++	*Staphylococcus* sp.
2.	MH-2	MH-I	JQ266327	++	*Micrococcus luteus*
3.	MH-3	MH-I	JQ266314	++	*Halotelea alkalienta*
4.	MH-6	MH-I	JQ266333	++	*Exiguobacterium* sp.
5.	MH-7	MH-I	JQ266330	+	*Microbacterium* sp.
6.	MH-8	MH-I	JQ266322	+	*Bacillus* sp.
7.	MH-9	MH-I	JQ266323	+	*Bacillus* sp.
8.	MI-10	MI-I	JQ266313	++	*Halotalea alkalilenta*
9.	MH-11	MH-I	JQ266328	++	*Curtobacterium* sp.
10.	MH-12	MH-I	JQ266309	++	*Pseudomonas* sp.
11.	MI-13	MI-I	JQ266326	++	*Microbacterium* sp.
12.	MI-14	MI-I	JQ266315	+	*Halotalea alkalilenta*
13.	MI-15	MI-I	JQ266312	++	*Halotalea alkalilenta*
14.	MI-16	MI-I	JQ266316	++	*Halotalea alkalilenta*
15.	MI-17	MI-I	JQ266331	++	*Curtobacterium* sp.
16.	MI-18	MI-I	JQ266336	++	*Erwinia* sp.
17.	MI-19	MI-I	JQ266318	++	*Staphylococcus* sp.
18.	MI-23	MI-I	JQ266310	++	*Pseudomonas* sp.

Table 2. Isolate IDs, Genbank Accession numbers and Blast identification of bacteria isolated from mango trees in Tando Jam, Pakistan.

No.	Isolate ID	Sample ID	Genbank accession	Colony PCR	BLAST identification
19	TJI- 7	TJI-III	JQ266311	++	*Pseudomonas* sp.
20	TJI-30	TJI-III	JQ266334	++	*Enterobacter* sp.
21	TJI-31	TJI-III	JQ266329	++	*Microbacterium* sp.
22	TJI-33	TJI-III	JQ266308	++	*Enterobacter* sp.
23	TJI-35	TJI-III	JQ266304	++	*Burkholderia* sp.
24	TJI-36	TJI-III	JQ266305	++	*Acinetobacter* sp.
25	TJI-38	TJI-III	JQ266325	++	*Microbacterium* sp.
26	TJI-39	TJI-I	JQ266303	++	*Enterobacter* sp.
27	TJI-42	TJI-I	JQ266324	++	*Bacillus* sp.
28	TJI-47	TJI-I	JQ266317	++	*Acinetobacter* sp.
29	TJI-48	TJI-I	JQ266332	+	*Exiguobacterium* sp.
30	TJI-49	TJI-I	JQ266335	++	*Burkholderia* sp.
31	TJI-51	TJI-I	JQ266307	++	*Acinetobacter* sp.
32	TJI-54	TJI-I	JQ266306	++	*Acinetobacter* sp.
33	TJI-55	TJI-I	JQ266321	++	*Lysinibacillus* sp.
34	TJI-56	TJI-I	JQ266320	++	*Lysinibacillus* sp.

In the leaf sample MH-1 from a healthy looking tree in Karachi *Bacillus*, *Micrococcus*, *Halotelea*, *Acinetobacter*, *Staphylococcus* sp., and *Exiguobacterium* sp., were detected. Plant associated and soil bacteria identified from MI-1 (leaf from dieback affected mango tree) were *Halotelea*, *Microbacterium*, *Erwinia* sp., *Pseudomonas* sp., *Curtobacterium*, and *Staphylococcus*. Both Karachi samples provided 4 colonies of *Halotelea alkalilenta*. The growth of this bacterium in high glucose and salt concentration was in agreement with published data (Ntougias *et al*., 2007). The bacterial colonies obtained from sample TJI-1, which was a bark sample from a dieback affected tree in Tando Jam, were identified as *Acinetobacter*, *Exiguobacterium*, *Lysinibacillus*, *Pseudomonas* sp., *Burkholderia* sp., *Enterobacter* sp., and *Staphylococcus*. The bacteria isolated from sample TJI-3 (bark from another dieback affected mango tree) were *Microbacterium*, *Pseudomonas* sp., *Enterobacter* sp., *Burkholderia* sp. and *Acinetobacter*. It was noted that species of *Pseudomonas*, *Burkholderia*, *Erwinia*, *Pantoea* and *Enterobacter* are established plant pathogens (Jackson, 2009). Hence their identification in dieback affected mango trees suggests a putative involvement in the disease. *Pseudomonas* and *Burkholderia* have ecologically distinct species including both non pathogenic (*P. putida*, *P. fluorescens* and *B. tuberum)* as well as plant pathogenic species (*P. syringae*, *P. viridiflava* and *B. cenocepacia*) (Compant *et al*., 2008). *Pseudomonas* and *Burkholderia* also contain the cross kingdom pathogens having capability to infect both animals and plants (Rahme *et al*., 1995; Rahme *et al*., 2000). *Pseudomonas syringae* is known to cause apical necrosis and black spot infection in mango tree (Cazorla *et al*., 1998). It is interesting to note that inoculation of sissoo seedlings with *Pseudomonas* isolates from dieback affected sissoo trees in Bangladesh produced typical disease symptoms (Tantau *et al*., 2011). *Erwinia amylovora* is causative agent of fire blight, a necrotic infection of apples and pears (Boureau *et al*., 2006). *Pantoea stewartii* is responsible for Stewart's wilt, a serious disease of *Zea mays* (Wensing *et al*., 2010; Morohoshi *et al*., 2007). The genus *Acinetobacter* contains numerous plant pathogenic species (Jackson, 2009). *Enterobacter pyrinus* causes brown leaf spot diseases of pear trees **(**Chung *et al*., 1993; Jackson, 2009). The *Curtobacterium* species cause several infections of legumes, solanaceous and monocot plants including wheat and maize (Jackson, 2009; Collins & Jones, 1982; Chen *et al*., 2007). Moreover, a thermotolerant acetic acid bacterium *Acetobacter senegalensis* has been isolated in Senegal (sub-Saharan Africa) from mango fruit (Ndoye *et al*., 2007).

The genera-level characterization through partial 16S rDNA sequences revealed the association of these diverse bacterial populations with infected mango trees in southern Pakistan. This study provided an impetus for in-depth species-level characterization and mode of association of these bacteria with infected mango trees.

References

Ah-You, N., Gagnevin, L., Chiroleu, F., Jouen, E., Neto, J.R. and O. Pruvost. 2007. Pathological variations within Xanthomonas campestris pv. mangiferaeindicae support its separation into three distinct pathovars. *Phytopathol.,* 97(12): 1568-1577.

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W. and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.,* 215: 403-410.

Arrebola, E., Cazorla, F.M., Codina, J.C., Gutiérrez-Barranquero, J.A., Pérez-García, A. and A. de Vicente. 2009. Contribution of mangotoxin to the virulence and epiphytic fitness of Pseudomonas syringae pv. syringae. *Intern. Microbiol.,* 12: 87-95

Baksha, M.W. and A.C. Basak. 2003. Mortality of Sissoo (*Dalbergia sissoo* Roxb.) and its management in Bangladesh. In: Mortality of Sissoo (*Dalbergia sissoo*) and Top Dying of Sundri (*Heritiera fomes*) in Bangladesh, (Ed.): Baksha M.W. pp. 1-6. Bangladesh Forest Research Institute, Chittagong.

Boureau, T., H. ElMaarouf-Bouteau, A. Garnier, M.N. Brisset, C. Perino, I. Pucheu and M.A. Barny. 2006. DspA/E, a type III effector essential for Erwinia amylovora pathogenicity and growth in planta, induces cell death in host apple and nonhost tobacco plants. *Mol. Plant Microbe Interact.,* 19(1):16-24.

Cazorla, F.M., J.A. Tores, L. Olalla, A. Perez-Garcia, J.M. Farre and A. Vicente. 1998. Bacterial apical necrosis of mango in southern Spain: a disease caused by *Pseudomonas syringae* pv. *syringae*. *Phytopathol.,* 88: 614-620.

Chen, Y.F., Y.N. Yin, X.M. Zhang and J.H. Guo. 2007. *Curtobacterium flaccumfaciens pv. beticola*, a new pathovar of pathogens in sugar beet*. Plant Dis.,* 91(6): 677-684.

Chung, Y.R., D.J. Brenner, A.G. Steigerwalt, B.S. Kim, H.T. Kim and K.Y. Cho. 1993. *Enterobacter pyrinus* sp. nov., an organism associated with brown leaf spot disease of pear trees. *Int. J. Syst. Bacteriol.,* 43**:** 157-161.

Collins, M.D. and D. Jones. 1982. Reclassification of *Corynebacterium pyogenes (Glage)* in the genus *Actinomyces*, as *Actinomyces pyogenes* comb. nov. *J. Gen. Microbiol.,* 128: 901-903.

Compant, S., Nowak, J., Coenye, T., Clement, C. and E.A. Barka. 2008. Diversity and occurrence of *Burkholderia* spp., in the natural environment. *FEMS Microbiol. Rev.,* 32: 607-626.

Freeman, S., M. Maimon and Y. Pinkas. 1999. Use of GUS transformants of *Fusarium subglutinans* for determining etiology of mango malformation disease. *Phytopathol.,* 89: 456-461.

Haggag, W.M. and M.E. Abd El-Wahab. 2009. First report of *Fusarium sterilihyphosum*- and *F. proliferatum*-induced malformation disease of mango in Egypt. *J. Plant Pathol.,* 91(1): 231-240.

Hartung, F., R. Werner, M.I. Hoque, S.S. Alam, S. Khan, A.R. Paul and H.-P. Mühlbach. 1998. Association of phytopathogenic bacteria with top-dying disease of sundri tree (Heritiera fomes) in Bangladesh. *Angew. Bot.,* 72: 48-55.

Iqbal, Z., Mehboob-ur-Rahman, A.A. Dasti, A. Saleem and Y. Zafar. 2006. RAPD analysis of *Fusarium* isolates causing ''Mango Malformation'' disease in Pakistan. *World J. Microbiol. Biotechnol.,* 22: 1161-1167.

Jackson, W.R. 2009. Plant Pathogenic Bacteria: Genomics and Molecular Biology. Caister Academic Press, Norfolk.

Knight, R.J.R. 1980. Origin and world importance of tropical and subtropical fruit crops. In: *Tropical and Subtropical Fruits*. (Eds.): S. Nagy, P.E. Shaw. AVI, Westport, CT, USA.

Krishna, H. and S.K. Singh. 2007. Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement: a review. *Biotechnol. Adv.,* 25: 223-243.

Lim, T.K. and K.C. Khoo. 1985. Diseases and disorders of mango in Malaysia. Tropical Press, Kuala Lumpur.

Litz, R.E. 2009. The mango botany, production and uses (2nd edition) CBI International Wallingford.

Morohoshi, T., Y. Nakamura, G. Yamazaki, A. Ishida, N. Kato and T. Ikeda. 2007. The plant pathogen *Pantoea ananatis* produces *N*-Acylhomoserine lactone and causes center rot disease of onion by quorum sensing. *J. Bacteriol.*, 189(22): 8333-8338.

Mraz, I., I. Pankova, K. Petrizik and M. Sip. 1999. Erwinia and Pseudomonas bacteria can be reliably screened by an improved serological agglutination test. *J. Phytopathol.*, 147: 429-431.

Ndoye, B., I. Cleenwerck, K. Engelbeen, R. Dubois-Dauphin, A.T. Guiro, S. Van Trappen, A. Willems and P. Thonart. 2007. Acetobacter senegalensis sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (*Mangifera indica* L.). *Int. J. Syst. Evol. Microbiol.*, 57: 1576-1581.

Ntougias, S., G. Zervakis and C. Fasseas. 2007. *Halotalea alkalilenta* gen. nov., sp. nov., a novel osmotolerant and alkalitolerant bacterium from alkaline olive mill wastes, and emended description of the family Halomonadaceae Franzmann *et al*., 1989, emend. Dobson & Franzmann 1996. *Int. J. Syst. Evol. Microbiol.*, 57: 1975-1983.

Ploetz, R.C. 2003. Diseases of mango. In: *Diseases of Tropical Fruit Crops.* (Ed.): R.C. Ploetz. CAB International, Wallingford, pp 327-363.

Pruvost, O., P. Roumagnac, C. Gaube, F. Chiroleuand and L. Gagnevin. 2005. New media for the semiselective isolation and enumeration of Xanthomonas campestrispv. mangiferaeindicae, the causal agent of mango bacterial black spot. *J. Appl. Microbiol.*, 99: 803-815.

Rahme, L.G., E.J. Stevens, S.F. Wolfort, J. Shao, R.G. Tompkins and F.M. Ausubel. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. *Science*, 268(5219): 1899-1902.

Rahme, L.G., F.M. Ausubel, H. Cao, E. Drenkard, B.C. Goumnerov, G.W. Lau, S. Mahajan-Miklos, J. Plotnikova, M.W. Tan, J. Tsongalis, C.L. Walendziewicz and R.G. Tompkins. 2000. Plants and animals share functionally common bacterial virulence factors. *Proc. Natl. Acad. Sci. U. S. A.*, 97(16): 8815-8821.

Rajput, K.S. and K.S. Rao. 2007. Death and decay in the trees of Mango (*Mangifera indica* L.). *Microbiol. Res.*, 162: 229-237.

Schaad, N.W., R.D. Frederick, J. Shaw, W.L. Schneide, R. Hickson, M.D. Petrillo and D.G. Luster. 2003. Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annu. Rev. Phytopathol.*, 41: 305-324.

Staden, R. 1996. The staden sequence analysis package. *Mol. Biotechnol.*, 5: 233-236.

Tantau, H., M.I. Hoque, R.H. Sarker and H.-P. Mühlbach. 2005. 16S rDNA sequence analysis of bacterial isolates from die-back affected Sissoo trees (*Dalbergia sissoo* Roxb.) in Bangladesh. *J. Phytopathol*., 153: 517-521.

Tantau, H., S. Renk, D. Schultz, H. Meyer, J. Schulze, D. Palm, A. Stubbe, N. Valdez, R.H. Sarker, S.S. Alam, M.L. Saha, M.S. Khan, M.I. Hoque and H.-P. Mühlbach. 2011. Infectivity assays and sequence analyses for unassigned *Pseudomonas* species as putative cause of dieback disease of *Dalbergia sissoo* Roxb. in Bangladesh. *Plant Tissue Cult. Biotech.*, 21(2): 101-113.

Wensing, A., S. Zimmermann and K. Geider. 2010. Identification of the corn pathogen Pantoea stewartii by mass spectrometry of whole-cell extracts and its detection with novel PCR primers. *Appl. Environ. Microbiol*., 76(18): 6248-6256.

Young, J.P.W., H.L. Downer and B.D. Eardly. 1991. Phylogeny of the phototrophic Rhizobium strain BTAi1 by polymerase chain reaction based sequencing of a 16S rRNA gene segment. *J. Bacteriol*., 173: 2271-2277.

(Received for publication 9 January 2013)