Journal of Plant Protection Research ISSN 1427-4345

RAPID COMMUNICATION

Bacterial watermark disease on *Salix alba* caused by *Brenneria salicis* in Iran

Esmaeil Basavand[1], Pejman Khodaygan[1]*, Mojtaba Dehghan-Niri[2], Saman Firouzianbandpey[1]

[1] Department of Plant Pathology, Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran

[2] Department of Plant Pathology, Ferdowsi University of Mashhad, Mashhad, Iran

Vol. 61, No. 2: 195–199, 2021

DOI: 10.24425/jppr.2021.137027

Received: November 8, 2020
Accepted: January 20, 2021

*Corresponding address:
pkhodaygan@vru.ac.ir

Abstract

In 2016, bacterial canker symptoms, often with dried ooze, were observed on *Salix alba* plants in municipal lands and parks in Kerman and Fars provinces, Iran. To determine the causative agent, samples were collected from symptomatic trees, and macerates of the affected bark tissues were plated on sucrose nutrient agar (SNA). Ten isolates were identified by phenotypic characterization, pathogenicity tests, and two of them further confirmed identity using sequence analysis of the partial of 16S rRNA and *gyrB* genes, and phylogenetic analysis. The isolates showed the highest identity (99–100%) with *Brenneria salicis*. To our knowledge, this is the first report of watermark disease on *S. alba* caused by *B. salicis* in Iran.

Keywords: *Brenneria salicis*, *Enterobacteriaceae*, white willow, Multilocus sequencing analysis

White willow (*Salix alba*) is a medicinal plant native to Europe. In the past, watermark disease (WMD) has been described in various countries such as, England, the Netherlands, Belgium and Japan (Huvenne *et al*. 2009; Grosso *et al*. 2011). 'Watermark' refers to the high moisture content of the wood caused by disturbances in water conductivity (Huvenne *et al*. 2009). It is one of the most important bacterial diseases of willow (Meas *et al*. 2002). The causal agent of WMD in *Salix* spp. is the Gram-negative facultative anaerobic bacterium, *Brenneria salicis* (Hauben *et al*. 1998; Meas *et al*. 2009).

During the spring of 2016, canker like symptoms were observed on white willow trees in municipal lands and parks in Kerman and Fars provinces, Iran (with incidence of 20%). The diseased plants had wilting branches and cankers on the stem (Fig. 1A) and trunk which almost always were associated with dried ooze (Fig. 1B, C). There were brown to black lesions under the cankers. Samples (a total of 10 infected samples from 10 plants) from branches of infected white willows were collected and transferred to a laboratory of phytobacteriology. Isolation of bacteria was done according to conventional bacteriological methods (Borkar 2017). Briefly, the segments of infected tissues were crushed in mortars with 10 ml of sterile distilled water (SDW). Loopfuls of the resulting suspensions were plated on sucrose nutrient agar (SNA), and the plates were incubated at 26 ± 1°C for 2–4 days. Ten isolates of the bacterium were obtained and they were described phenotypically, according to the standard microbiological methods described by Schaad *et al*. (2001). The colonies were 1–2 mm in diameter on SNA medium after 2 days at 26 ± 1°C, with an entire margin, and were circular, convex and shiny. The bacterial isolates were kept at –80°C in a (40% v/v) glycerol solution. Based on the phenotypic characterization, bacterial isolates in the present study formed a homogeneous group. The characteristics of these isolates such as no growth at 37°C, arginine dihydrolase, hydrolysis

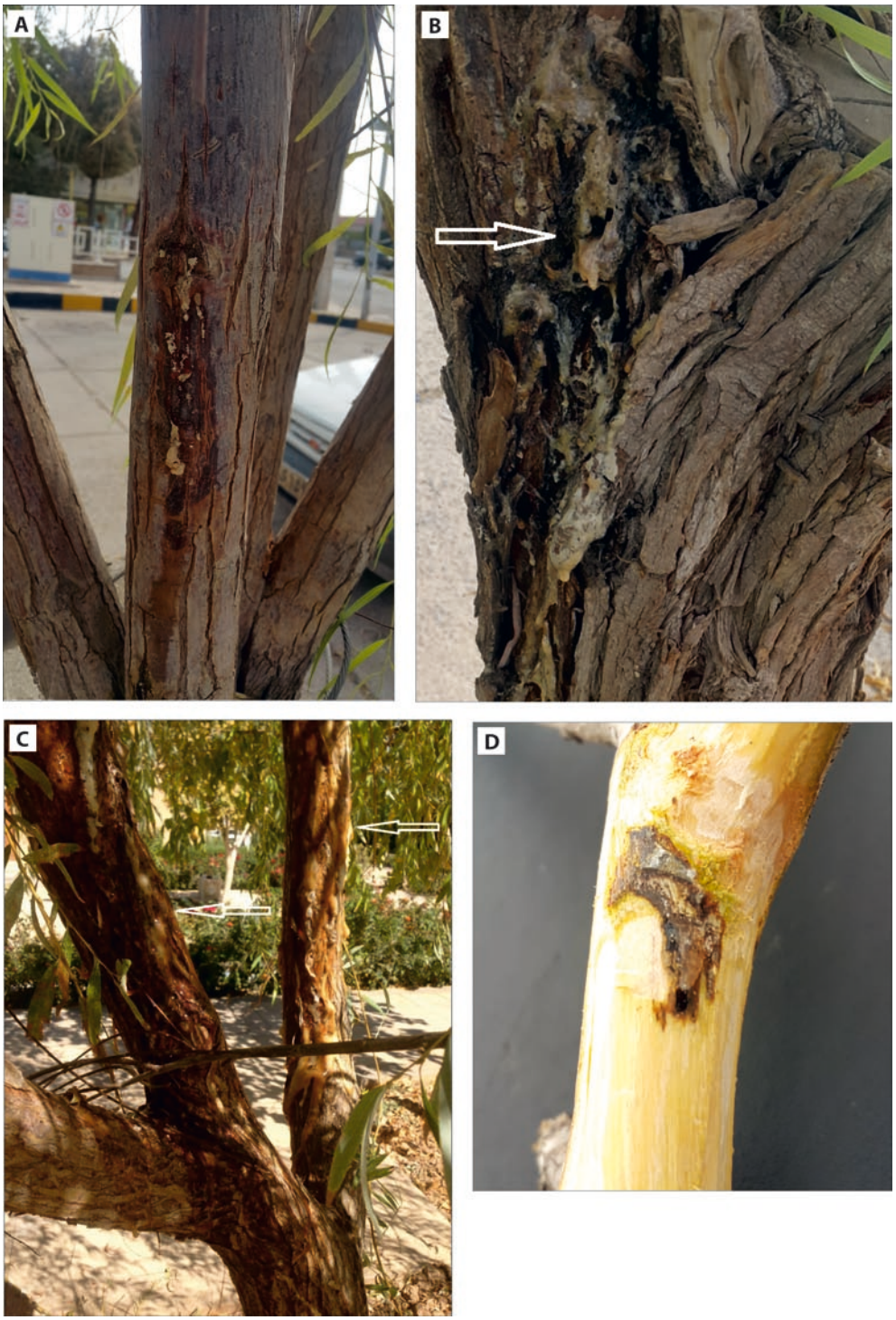

Fig. 1. Symptoms of watermark disease on white willow (*Salix alba*) caused by *Brenneria salicis* in Iran: A – bark canker symptoms with dried ooze on the stem under natural conditions; B – typical wounds on the trunk; C – brownish and sticky liquid ooze from wounds in the bark; D – dark lesion observed 2 months after inoculation of a bacterial suspension of isolate BI1 on stem of white willow (1-year-old)

of starch, gelatin and arbutin, utilization of cellobiose and tartrate, acid production from sucrose, glucose and mannitol, corresponded well with *B. salicis*, except tests for D-galactose and D-lactose (Sakamoto *et al.* 1999; Denman *et al.* 2012). The phenotypic and biochemical characteristics of our willow isolates, and reference strains of *B. salicis* that were pathogenic to white willow plants, are compared in Table 1. Inoculation of bacterial suspensions (all 10 bacterial isolates) containing approximately 10^7 colony-forming units per milliliter (CFU · ml^{-1}), into geranium (*Pelargonium* × *hortorum*) gave a positive hypersensitive reaction (Fig. 2) (Klement *et al.* 1990). The leaves infiltrated with sterile water served as a negative control. Under

Table 1. Biochemical and phenotypic features of 10 bacterial isolates from watermark disease of white willow (*Salix alba*) in Iran, and *Brenneria salicis* strains taken from Sakamoto *et al*. 1999, and Denman *et al.* 2012

Characteristics	Present isolates*	*Brenneria salicis***	Organic compound	Present isolates	*Brenneria salicis*
Gram reaction	–	–	D-Xylulose	–	ND
Oxidase	–	–	D-Sorbitol	–	–
Catalase	+	+	D-Lactose	–	+
Oxidative/fermentative	+	+	L-Arabinose	–	–
Levan	+	+	D-Fructose	+	+
NaCl 2% tolerance	+	+	Sucrose	+	+
Fluorescent pigment	–	–	D-Glucose	+	+
Pigment in EMB	+	ND	D-Galactose	+	–
Esculin hydrolysis	+	+	Mannitol	+	+
Starch hydrolysis	–	–	Raffinose	+	+
Gelatin hydrolysis	–	–	L-Ornithine	–	–
Arbutin hydrolysis	–	–	L-Arginine	–	–
Tween 80 hydrolysis	–	–	L-Tartrate	–	–
Urease	–	–	Glycerol	+	+
Nitrate reduction	+	+	Maltose	–	–
Arginine dihydrolase	–	–	Cellobiose	–	–
H_2S from cysteine	+	+			
H_2S from peptone	+	+			
Indole production	–	–			
Potato soft rot	–	–			
Tyrosinase	–	–			
Phenylalanine deaminase	–	–			
Growth at 37°C	–	–			

(+) – positive reaction; (–) – negative reaction; ND – not determinate
*the author's data; **data from Sakamoto *et al*. 1999, and Denman *et al*. 2012

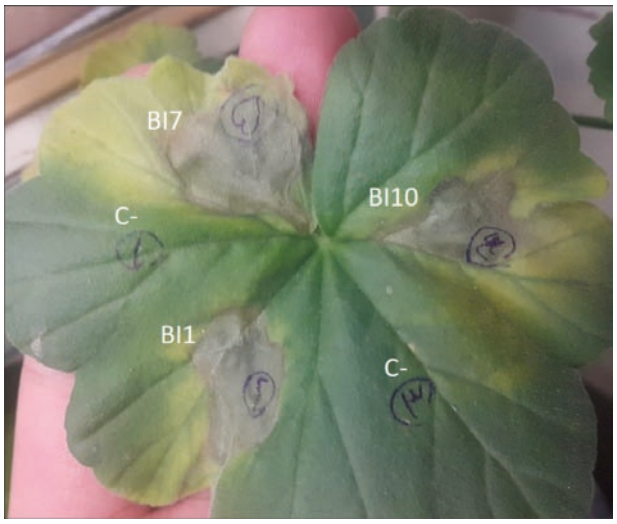

Fig. 2. Hypersensitive reaction (HR) on geranium (*Pelargonium* × *hortorum*) observed 24 h after inoculation of *Brenneria salicis* isolates (BI1, BI7 and BI10); (C-) – negative control (distilled water inoculation)

glasshouse conditions (25°C and 60% humidity), pathogenicity of all 10 isolates was confirmed by injecting 100 µl of bacterial suspension (1×10^7 CFU · ml^{-1}) using a sterile syringe into young twigs of white willow plants (1-year-old) (Khodaygan and Habibi 2019). Sterile distilled water was used as a negative control. Dark lesions were observed 2 months after inoculation, which enlarged in size and changed color with time (Fig. 1D). The negative control (distilled water inoculation) did not develop any symptoms. To fulfill Koch's postulates, re-isolations performed on SNA medium from seedlings with symptoms consistently yielded typical transparent-to-white-colored bacterial colonies that were identical in appearance to those used for the inoculations. These isolates were identified as *B. salicis* based on colony morphology and phenotypic characteristics and showed pathogenicity when re-inoculated into white willow plants (1-year-old). One of the isolates was deposited in the Culture

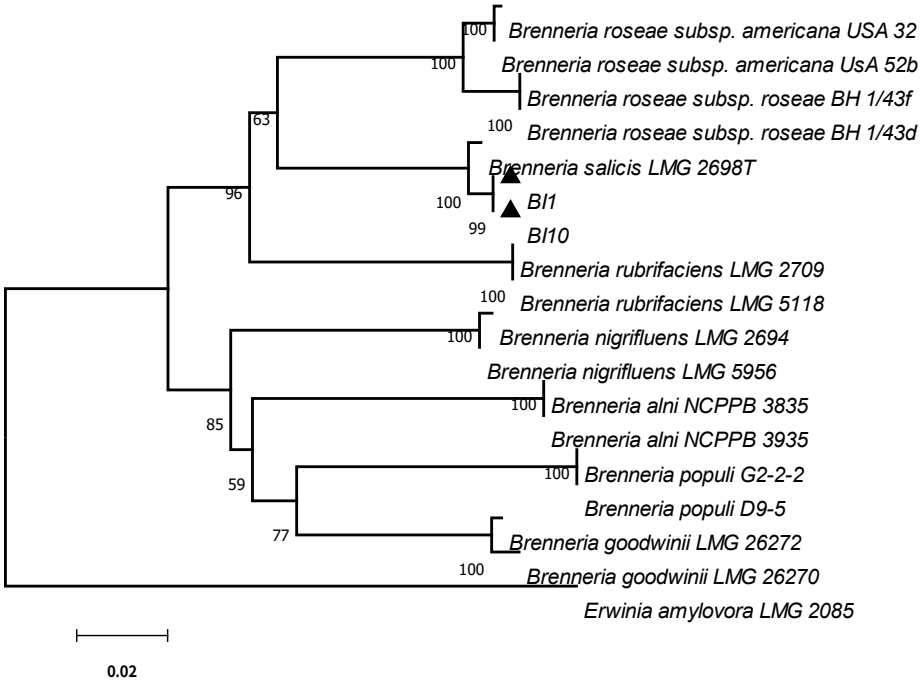

Fig. 3. Neighbor-joining phylogeny of *gyrB* gene in *Brenneria salicis* strains obtained in this study. This indicates the relationship among the isolates BI1 and BI10 obtained from watermark disease in Kerman and Fars provinces of Iran and *Brenneria* species. Numbers on the branches indicate bootstrap values. *Erwinia amylovora* strain LMG 2085 was used to root the tree

Collection of Microorganisms at the Vali-E-Asr University of Rafsanjan (VRU 1864), Iran. Two representative pathogenic isolates were chosen to perform molecular identification. Genomic DNAs were extracted by the CTAB (cetyltrimethylammonium bromide) method with slight modification (Ausuble *et al*. 1992; Basavand *et al*. 2020). Universal primer pairs FD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and RD1 (5′-AAGGAGGTGATCCAGCC-3′) were used for the 16S rRNA (Weisburg *et al*. 1991). Furthermore, the partial *gyrB* gene (DNA gyrase subunit B) was amplified using primer pairs reported by Brady *et al*. (2008). The *gyrB* gene was chosen because it has been widely used to resolve the phylogeny of *Enterobacteriales* members. Furthermore, it is often used as a reference gene to accurately identify *Brenneria* species (Brady *et al*. 2012) The polymerase chain reaction (PCR) was done using a T-100 (Applied Biosystem) thermal cycler in 25 µl final volume mixtures containing 12.5 µl 2X *Taq* DNA PCR master mix (Odense, Denmark), 0.2 µM of each primer and 2 µl of template DNA. The 16S rRNA, and *gyrB* genes were amplified according to Weisburg *et al*. (1991) and Brady *et al*. (2008), respectively. The PCR products were visualized on 1.5% agarose gel. Fragments of 1400 bp for 16S rRNA and 744 bp for *gyrB* were sequenced by Macrogen Corporation, Daejeon (South Korea).

The nucleotide sequences (GenBank accession numbers MT320015 and MT320019 for 16S rRNA, MW447112 and MW447113 for *gyrB*) were compared with those deposited in NCBI database by the BLAST software. Sequence analyses demonstrated that the isolates have highest similarity (99–100%) with *B. salicis*. Sequences of *Brenneria* species were taken from the GenBank and a phylogenetic tree was constructed based on the 16S rRNA and *gyrB* using MEGA 7.0 with the Neighbor joining method (Kumar *et al*. 2016). Consequently, phylogenetic analysis of the 16S rRNA gene sequences confirmed the strains as *B. salicis* (data not shown). Furthermore, the phylogenetic analysis using the *gyrB* (Fig. 3) confirmed that the representative isolates (BI1 and BI10) were phylogenetically within the *B. salicis* clade. Taken together, these data reveal that the isolated strains which cause watermark disease on *S. alba* trees are *B. salicis*. To our knowledge, this is the first report of *B. salicis* as the cause of bacterial watermark disease of *S. alba* in Iran. The high prevalence of watermark disease has caused severe damage to *S. alba* in Iranian municipal lands and parks. Therefore, more research on the pathogen and management of the disease is required.

Acknowledgements

This research was supported by the Vice Chancellor of Research and Technology grant (AGR95PP5938) at the Vali-E-Asr University of Rafsanjan, Iran.

References

Ausuble F., Brent F.M., Kingestone R.E., Moor D.D., Smith J.A., Seideman J.G., Struhl K. 1992. Current Protocols in Molecular Biology. Greene, Publishing Associates, Wiley Interscience, New York, USA, 4648 pp.

Basavand E., Khodaygan P., Babaeizad V., Rahimian H., Mirhosseini H. 2020. Soft rot disease caused by *Klebsiella aerogenes* on *Austrocylindropuntia subulata* in Iran. Indian Phytopathology 73: 371–372. DOI: https://doi.org/10.1007/s42360-020-00201-6

Borkar S.G. 2017. Laboratory Techniques in Plant Bacteriology. CRC Press, New York, USA, 320 pp.

Brady C.L., Cleenwerck I., Denman S., Venter S.N., Rodríguez-Palenzuela P., Coutinho T.A., De Vos P. 2012. Proposal to reclassify *Brenneria quercina* (Hildebrand and Schroth 1967) Hauben *et al*. 1999 into a new genus, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov., descriptions of *Lonsdalea quercina* subsp. *quercina* comb. nov., *Lonsdalea quercina* subsp. *iberica* subsp. nov. and *Lonsdalea quercina* subsp. *britannica* subsp. nov., emendation of the description of the genus *Brenneria*, reclassification of *Dickeya dieffenbachiae* as *Dickeya dadantii* subsp. *dieffenbachiae* comb. nov., and emendation of the description of *Dickeya dadantii*. International Journal of Systematic and Evolutionary Microbiology 62 (7): 1592–1602. DOI: https://doi.org/10.1099/ijs.0.035055-0

Brady C., Cleenwerck I., Venter S., Vancanneyt M., Swings J., Coutinho T. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Systematic and Applied Microbiology 31: 447–460. DOI: https://doi.org/10.1016/j.syapm.2008.09.004

Denman S., Brady C.L., Kirk S., Cleenwerck I., Venter S.N., Coutinho T.A., De Vos P. 2012. *Brenneria goodwinii* sp. nov., associated with acute oak decline in the UK. International Journal of Systematic and Evolutionary Microbiology 62: 2451–2456. DOI: https://doi.org/10.1099/ijs.0.037879-0

Grosso S., Mason G., Ortalda E., Scortichini M. 2011. *Brenneria salicis* associated with watermark disease symptoms on *Salix alba* in Italy. Plant Disease 95 (5): 772. DOI: https://doi.org/10.1094/PDIS-11-10-0781

Hauben L., Moore E.R.B., Vauterin L., Steenackers M., Mergaert J., Verdonck L., Swings J. 1998. Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. Systematic and Applied Microbiology 21 (3): 384–397.

Huvenne H., Messens E., Maes M. 2009. Willow wood sap promotes the density-dependent pathogenesis of *Brenneria salicis*. Environmental Microbiology 11 (6): 1463–1472.

Khodaygan P., Habibi H. 2019. *Brenneria nigrifluens*, a latent threat for walnut trees in Alborz province of Iran. Iranian Journal of Plant Pathology 54 (4): 337–341.

Klement Z., Rudolph K., Sands D.C. 1990. Methods in Phytobacteriology. Akademiai, Kiado, Budapest, 568 pp.

Kumar S., Stecher G., Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33 (7): 1870–1874. DOI: https://doi.org/10.1093/molbev/msw054

Meas M., Baeyen S., De Croo H., De Smet K., Steenackers M. 2002. Monitoring of endophytic *Brenneria salicis* in willow and its relation to watermark disease. Plant Protection Science 38: 528–530. DOI: https://doi.org/10.17221/10545-PPS

Meas M., Huvenne H., Messens E. 2009. *Brenneria salicis*, the bacterium causing watermark disease in willow, resides as an endophyte in wood. Environmental Microbiology 11 (6): 1453–1462. DOI: https://doi.org/10.1111/j.1462-2920.2009.01873.x

Sakamoto Y., Takikawa Y., Sasaki K. 2002. Occurrence of watermark disease of willows in Japan. Plant Pathology 48 (5): 613–619. DOI: https://doi.org/10.1046/j.1365-3059.1999.00368.x

Schaad N.W., Jones J.B., Chun W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. APS Press, Minnesota, USA, 410 pp.

Weisburg W.G., Barns S.M., Pelletier D.A., Lane D.J. 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173 (2): 697–703.