Isolation and Characterization of a *Brenneria* sp. Shown to Be the Causal Agent of Bleeding Canker Disease of *Populus nigra* in Iran

Yousef Moradi-Amirabad[a], Gholam Khodakaramian[a, *], and Teresa Coutinho[b]

[a] *Department of Plant Protection, Faculty of Agriculture, Bu-Ali Sina University, Hamadan, Iran*
[b] *Department of Biochemistry, Genetics and Microbiology, Centre for Microbial Ecology and Genomics/Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa*
*e-mail: Khodakarami an@yahoo.com

Abstract—In 2017, cankers were observed on mature *Populus nigra* L. trees in Iran. The disease was characterized by copious amounts of brown exudation from cankers on the trunk, crotch and pruning scars in spring, summer and early autumn. Upon removal of the bark, reddish/brown lesions were visible in the woody tissue of the host. These symptoms were similar to those described on the same host in Spain. Isolations were performed from infected tissue. Pale cream bacterial colonies were consistently isolated from the infected tissue. The isolates were phenotypically identical to members of Pectobacteriaceae specifically *Brenneria* spp. The nucleotide sequences of the 16S rRNA and house-keeping genes, *gyrB*, *infB*, *rpoB*, and *gapA* indicated that the isolates from the trees in Iran were similar to *Brenneria salicis*, but formed a distinct cluster on their own suggesting that this was a new species of *Brenneria*. Pathogenicity tests were performed by inoculation of stems, trunks and shoots of age height where, poplar. At the site of inoculation, all isolates produced necrotic lesions and exudates 30 days after inoculation. Re-isolations and confirmation of their identity were performed fulfilling Koch's postulates.

INTRODUCTION

Black poplar (*Populus nigra* L.) is found in large areas of Europe and Asia and is typically a pioneer species found in riverine areas [1]. In Iran it is of both social and economic interest. It is planted as windbreaks for the protection of agricultural land, as ornamentals and for wood production [2].

In 2017, a bark canker with copious brownish exudations was observed on mature black popular trees in Hamadan province, Iran. The exudates were observed on the trunk, crotch or pruning scars in spring, summer and early autumn. When the bark was removed, reddish lesions within the woody tissue was observed (Fig. 1).

Diseases, especially bark cankers, reduce the quantity and quality of poplar wood. Plant pathogenic bacteria are one of the most important causes of bark cankers on poplar trees. Bark canker symptoms have been observed on *Populus monilifera*, *P. nigra*, and *P. monilifera* in the Netherlands, Belgium, France, England, and Italy [3]. Similar symptoms were reported in Spain on *Populus × euramericana* and the causal agent described as *Brenneria* sp. [4]. Recently, the causal agent of this disease on this host was reported to be *Lonsdalea populi* in Spain [5], Hungary [6] and China

[7]. Bacterial bark canker caused by *Xanthomonas populi* has previously been reported on poplar trees in Europe and the USA [8]. To date, none of these bacteria have been reported from Iran.

The genus *Brenneria* was first described by Hauben et al. [9]. Almost all species of the genus are plant pathogens and cause blight, canker, wilt, and necrosis of deciduous trees, including oak, willow, and walnut [9–12]. There are species in this genus which can cause bark cankers with stem bleeding. These include *Brenneria nigrifluens* and *B. rubrifaciens* causing bark canker and deep bark canker of walnut, respectively [11, 13], *B. alni* causing bark canker of alder [14], *Brenneria salicis* causing watermark disease (WMD) on willow [15–17], and *Brenneria* sp. causing bark canker on poplar trees [4]. *Brenneria goodwinii* and *B. roseae* have been isolated from symptomatic oak and hornbeam tissues with stem bleeding and have found to be associated with acute oak decline (AOD) [18–20]. *B. goodwinii* was, however, recently reported to be one of the key causal agents of tissue necrosis characterizing AOD in Britain [12].

Considering that nothing is known about the causative bacteria of bark canker on poplar trees in Iran,

Fig. 1. Symptoms of bark canker on Poplar mature trees caused by *Brenneria* sp. in Iran. (a−d) The symptoms of bark canker on poplar trees in the field. (e) The symptoms caused by *Brenneria* strain YMAPO10 in the greenhouse on excised stems 4 days after inoculation. (f) Control water-cultured excised stems inoculated with SDW. (g) The symptoms caused by *Brenneria* strain YMA-PO10 in greenhouse on excised stems after 3 days after inoculation. (h) Control excised stems inoculated with SDW. (i) The symptoms caused by *Brenneria* strain YMAPO10 in greenhouse on shoots of poplar seedling after 4 days after inoculation. (j) The symptoms caused by *Brenneria* strain YMAPO10 in the greenhouse and on the trunk of poplar mature trees in the field 18 days after inoculation. (k) Control mature trees inoculated with SDW only in the field. (l) Control mature trees inoculated with *B. goodwinii* strain ICMP21594 in the field.

the aim of this study was to determine the causal agent of the bark canker disease of *Populus nigra* in Iran.

MATERIALS AND METHODS

Symptoms, Sampling and Bacterial Isolation

In 2017 and 2018, samples were collected from 16 symptomatic *P. nigra* trees in Hamadan province of Iran (Table 1). In 2017, a bleeding canker with abundant brownish exudates was observed on poplar trees in Iran. Exudation occurred particularly on the tree trunk from pruning cuts, bark cracks, and branch crotches. Bleed points occurred at 0.5 to 2.5 m above ground level. Active bleeding canker was usually seen from spring to early autumn. After removing the outer bark behind the flow of exudates on the trunk, a necrosis tissue was observed on the inner bark and sapwood (Figs. 1a−1d). Other symptoms on affected trees were chlorosis, defoliation, progressive loss of

Table 1. Isolation details from symptomatic poplar trees (*Populus nigra*) sampled in this study

Tree reference	Location and site type	GPS position of tree (northings and eastings)	Date sampled	Strain number
20170001	Hamadan, Bu Ali Sina University	34.79312406,48.48376043	21/05/2017	YMAP1**, YMAP2
20170002	Hamadan, Bu Ali Sina University	34.80409068,48.48247469	21/05/2017	YMAP3, YMAP4
20170003	Hamadan, Bu Ali Sina University	34.79919287,48.47893315	10/06/2107	YMAP5*, **, YMAP6
20170004	Hamadan, Heidareh	34.80230292,48.46369291	10/06/2017	YMAP7**, YMAP8
20170005	Hamadan, Asadabad	34.74720320,48.09529974	10/06/2017	YMAP9, YMAP10*, **
20170006	Hamadan, Nahavand	34.24272364,48.31218149	22/10/2017	YMAP11, YMAP12,
20170007	Hamadan, Ganjnameh	34.76254306,48.43880725	22/10/2017	YMAP13, YMAP14**
20170008	Hamadan, Imamzadeh Kuh	34.80526568,48.39143077	22/10/2017	YMAP15, YMAP16
20180001	Hamadan, Marianaj	34.82672469,48.47195864	15/05/2018	YMAP17, YMAP18
20180002	Hamadan, Simin	34.86855988,48.31776452	15/05/2108	YMAP19, YMAP20
20180003	Hamadan, Bu Ali Sina University	34.79477876,48.48306236	15/05/2108	YMAP21, YMAP22
20180004	Hamadan, Bu Ali Sina University	34.79652494,48.48267574	15/05/2108	YMAP23, YMAP24
20180005	Hamadan, Ganjnameh	34.76293792,48.43851721	15/05/2108	YMAP25*, ** , YMAP26
20180006	Hamadan, Imamzadeh Kuh	34.80539728,48.39161412	25/05/2108	YMAP27, YMAPO28
20180007	Hamadan, Abasabad	34.77948354,48.47392756	25/05/2108	YMAPO29, YMAPO30
20180008	Hamadan, Darreh Morad Beyg	34.74176660,48.50154280	25/05/2108	YMAPO31, YMAPO32

 * = Indicates isolate used for molecular tests.
** = Indicates isolate used in pathogenicity tests.
— All isolates were used for phenotypic and BOX-PCR.

vigor, but the death of the trees was not observed. Each sample consisted of a single panel of outer bark, inner bark and sapwood tissue. Also, two non-symptomatic *P. nigra* trees were sampled. The collected samples were placed in paper bags and transferred to Plant Pathology Laboratory of Bu Ali Sina University in an ice-box.

For each sample, the outer bark was removed due to the presence a large number of saprophytic microorganisms and the samples were then washed under tap water. For isolation of bacteria from diseased trees, the pieces of tissue (5 × 30 mm) were cut from the interface between diseased and healthy phloem and sapwood tissue using a sterile scalpel. For isolation of bacteria from healthy trees, the pieces of tissue (5 × 30 mm) were also cut from apparently healthy tissue.

Both diseased and healthy pieces were surface-disinfected individually by dipping in 70% ethanol for 2 min, and rinsed in three changes (3 min each) of sterile distilled water (SDW). Each piece was placed in a sterile petri-dish and further fragmented manually in 6 ml SDW using a sterile scalpel. The resulting suspension was transferred to a test tube and shaken for about 30 min at 25°C. A loop-full of the resulting suspension was streaked on nutrient agar (nutrient agar; Merck, Darmstadt, Germany) and incubated in the dark at 28°C for 7 days. Single dominant colonies were selected and sub-cultured on NA again for purification [20]. The isolates were kept at 4°C for the short

term and in 30% (v/v) glycerol at −80°C for long term storage [21].

Phenotypic Characteristics of Isolates Obtained from Poplar Trees

Phenotypic features of the bacterial strains were characterized based on standard bacteriological methods. Sensitivity to 3% KOH [22], oxidative/fermentative test [23] and the oxidase reaction [24] was determined for each isolate. Formation of levan, catalase production, hydrolysis of gelatin, aesculin and starch, production arginine dihydrolase, H_2S production from cysteine, nitrate reduction, urease production, and production of fluorescent pigment on King's B medium were performed according to the methods described by Schaad et al. [21]. In addition, assimilation of carbon sources was tested using the basal medium of Ayers et al. [25] supplemented with 0.1–0.3% of the carbon compounds.

Genotypic Characteristics of Bacterial Isolates from Symptomatic Trees DNA Preparation

Three bacterial isolates obtained from symptomatic trees (YMAP5, YMAP10, and YMAP25) were used for genotypic tests. Bacteria were cultured on NA medium and kept at 28°C for 24 h. Bacterial suspensions (108 CFU/ml) were prepared in SDW and lysed

Table 2. The sequences accession numbers of poplar strains of *Brenneria* obtained from this study

Species name	Strain	16S rRNA	*gyrB*	*infB*	*rpoB*	*gapA*
Brenneria sp.	YMAPO5	MN007189	MK512579	MK512583	MK512587	MN062292
Brenneria sp.	YMAP010	MN007190	MK512580	MK512584	MK512588	MN062293
Brenneria sp.	YMAPO25	MN007191	MK512582	MK512586	MK512590	MN062294

by the addition of 1:10 volume of 10% KOH. They were heated at 100°C for 5 min and then placed at 4°C until cooled. Lysates were centrifuged at $10000 \times g$ for 10 min, and the supernatants were used directly for PCR or stored at −20°C until used [26]. The quality of DNA was determined by electrophoresis on agarose gel [27].

BOX-PCR

BOX-PCR fingerprints were produced using BOX-A1R primer (5'-CTA CGGCAAGGCGAC-GCTGACG-3') [28]. The total volume of the reaction was 25 μL which contained 2.5 μL 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.4), 2.5 mM MgCl$_2$, 0.4 mM dNTPs, 0.2 μM BOXA1R primer, 1.25 U Taq DNA polymerase (CinnaGen, Iran) and 2 μL DNA. Amplification was performed in a Techne TC-512 thermal cycler with initial denaturation for 7 min at 94°C, followed by 35 amplification cycles consisting denaturation (94°C, 1 min), annealing (59°C, 1.5 min) and extension (72°C, 2 min), with a final extension at 72°C for 10 min [29]. The PCR products were separated by electrophoresis in a horizontal 1.5% agarose gel in 1X TBE buffer (100 mM Tris, 500 mM boric acid and 1 mM EDTA), at 80 V for 120 min and stained with ethidium bromide. A 1 Kb GeneRuler™ ladder (GeneRuler, Fermentas, Vilnius, Lithuania) was used as a base pair size marker. The banding patterns of isolates were visualized and photographed using a gel documentation system DigiDoc H101 (Iran).

16S rRNA and Multilocus Sequence Analysis (MLSA)

The 16S rRNA gene of isolates was amplified using primers fD1 and rP2 [30]. PCR mix and amplification were performed according to Moradi-Amirabad and Khodakaramian [26] using XP thermal cycler TC-XP-G (Bioer, Germany). DNA gyrase B sub-unit (*gyrB*), initiation translation factor 2 (*infB*), and RNA polymerase β subunit (*rpoB*) genes was amplified using the primers and conditions as described by Brady et al. [31]. Partial amplification of the glyceraldehyde-3-phosphate dehydrogenase A (*gapA*) gene was conducted as described by Ma et al. [32].

PCR products were electrophoresed in 1.2% agarose gel, stained with ethidium bromide and observed with gel documentation system DigiDoc H101 (Iran). The amplified fragments were sequenced by the dideoxy termination method by BIONEER Co. (South Korea) on both strands employing the primers used for amplification. The sequence data were trimmed with BioEdit version 7.2.5 program [33] and compared with those from GenBank with the BLASTN program [34]. Clustal W was used for creating the sequence alignments [35]. Neighbor-joining tree was constructed employing MEGA X [36]. Bootstrap analysis was performed to test the stability on randomly chosen sets of positions with 1000 replicates [37]. The sequence data were deposited in GenBank and accession numbers are presented in Table 2. The representative isolates were deposited a culture collection in the Plant Pathology Laboratory of Bu Ali Sina University.

Pathogenicity Tests

For pathogenicity tests, the representative isolates (YMAPO1, YMAPO5, YMAPO7, YMAPO10, YMA-PO14, and YMAPO25) were grown on NA for 48 h at 28°C (Table 1). Cells were suspended in SDW and their density adjusted to 10^7 CFU/mL. Four pathogenicity trials were undertaken. In the first trial, pathogenicity tests were performed on excised stems (2 cm in diameter and 30 cm in length) of *P. nigra* trees in a greenhouse (28°C incubator). The upper end of stems were wrapped with plastic film to prevent drying and lower cut surface of them were placed in saucers containing water. The stems were surface sterilized with 70% ethanol and cross-shaped wounds (5 mm long) were made in the bark with a sterile knife. 100 μL bacterial suspension was introduced into the wound with a sterile dropper. The inoculated segments were covered with polyethylene bags to maintain the humidity and were kept in darkness at 28°C [7]. In the second trial, the wound was made using a 10 mm cork-borer on excised stems of *P. nigra* trees. Half a loop of 24 h NA culture of each representative isolate was inserted into the wound and the stems placed in a greenhouse (28°C). The outer bark plug was pulled back into the inoculation point and wrapped with parafilm and sealed with duct tape. Inoculated stems were incubated at 25°C in the growth chamber with a 12 h photoperiod [12]. The third pathogenicity trial was conducted on shoots of poplar seedlings by injecting 50 μL bacterial suspension (107 CFU/mL) as described by Schaad et al. [21].

The forth pathogenicity trial was conducted in the field on mature trees of *P. nigra* at Bu-Ali Sina Univer-

sity, Hamadan where the disease first observed. Two cm long wounds were made in the trunk bark using a sterile knife. Two hundred ul of the bacterial suspension (10^7 CFU/mL) was introduced into the wound. The wounds were wrapped with parafilm and unwrapped after three days [7]. Also, the first, second, and third pathogenicity trials were conducted to assess the potential pathogenicity of isolates obtained from healthy trees. Inoculated stems were assessed for symptom daily. In all trials, control excised stems and plants were inoculated with SDW and *Brenneria goodwinii* ICMP21594. Each trial was conducted for each isolate in three repetitions. At the end of the trials, re-isolations were performed at the point of inoculation and/or at the interface between healthy and diseased tissue.

RESULTS

Sampling and Bacterial Isolation

From 16 symptomatic trees, a total of 32 bacterial strains were isolated from difference region of Hamadan province in Iran (Table 1). Only, *Brenneria*-like colony appeared on the culture medium, after 20 days. All of colonies were 1−2 mm in diameter on NA after 4 days at 20°C, pale cream, circular, convex, smooth with entire margins and shining (Fig. 2a). Colonies of all isolates had a metalic green sheen on EMB agar (Fig. 2b).

Phenotypic Characteristics of Bacterial Isolates from Symptomatic Trees

Strains were Gram-negative, and facultatively anaerobic. They were positive for catalase reaction, and hydrolysis of aesculin, and H_2S production, but negative for tobacco hypersensitivity reaction, oxidase, urease production, and lysine decarboxylase, fluorescent production on King B, starch hydrolysis, and indole production. All isolates produced acid from D-glucose, fructose, L-rhamnose, cellobiose, sucrose, maltose and D-mannitol, but they didn't produce acid from L-arabinose, lactose, D-raffinose, D-xylose, D-galactose, trehalose, adonitol, D-sorbitol, and dulcitol. Other phenotypic characteristics are summarized in Table 3.

Phenotypic Characteristics of Bacterial Isolates from Healthy Trees

Also, two healthy poplar trees were sampled and 17 bacterial isolates were obtained. They were mostly Gram-positive bacteria (eight strains), *Pseudomonas* spp. (five strains), and members of the *Enterobacteriales* (three strains). None of the isolates obtained from healthy tree were identified as *Brenneria* sp. (Table 4).
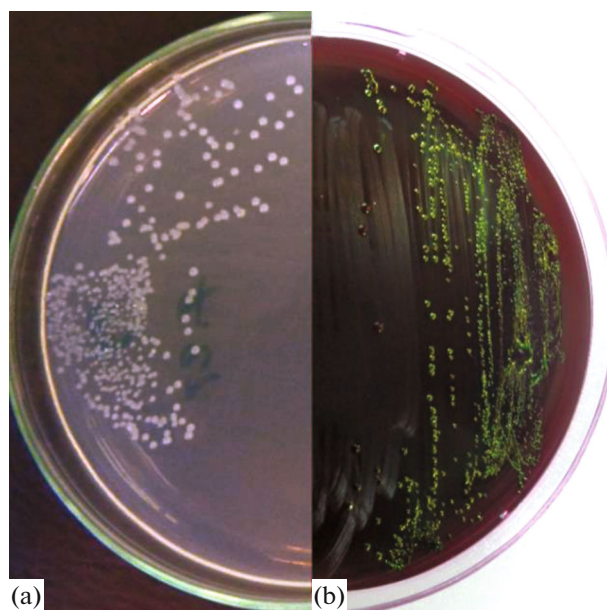

Fig. 2. Colonies of the *Brenneria* sp. from Iran (a) NA medium. (b) Eosin methylene-blue culture (EMB) medium showed metallic green colony colour.

GENOTYPIC CHARACTERISTICS OF BACTERIAL ISOLATES FROM SYMPTOMATIC TREES

BOX-PCR

The genomic fingerprinting pattern of symptomatic poplar isolates were generated using the BOXA1R primers. The amplified bands in BOX-PCR ranged from 350 to 3000 bp (Fig. 3). The genomic fingerprint pattern was re-generated upon repeat of the procedure. Poplar isolates were grouped into one group based on binding pattern in BOX-PCR. All of the poplar isolates obtained in this study showed same banding pattern based on BOX-PCR fingerprint and were placed in one group (Fig. 3).

16S rRNA Sequences Analysis

The three isolates (YMAPO5, YMAPO10, and YMAPO25) from symptomatic poplar trees demonstrate 100% 16S rRNA gene sequence pairwise similarity to each other and *Brenneria* strain NCPPB 4297, and 99.48 and 99.93% to NCPPB4296 and NCPPB4299, and 99.19 % to the type strains of *B. salicis* LMG 2698. The poplar isolates and NCPPB4296, NCPPB 4297 and NCPPB 4299 formed a single cluster with 91% bootstrap support on a separate branch in the 16S rRNA phylogenetic tree, and demonstrated a close phylogenetic relationship to *B. salicis* strain LMG 2698 (the type species of *Brenneria*) with 100% bootstrap in neighbour-joining (Fig. 4).

Table 3. Phenotypic characteristics distinguishing strains of *Brenneria* sp. isolated from symptomatic poplar trees in Iran and in comparison with described taxa of the genera *Brenneria* and *Lonsdalea*

Species[a]	1	2	3	4	5	6	7	8	9	10
Gram reaction	−[b]	−	−	−	−	−	−	−	−	−
Oxidative growth	+[c]	+	+	+	+	+	+	+	+	+
Fermentative growth	+	+	+	+	+	+	+	+	+	+
Oxidase	−	−	−	−	−	−	−	−	−	−
Urease production	−	−	−	−	−	−	−	−	−	−
Lysine decarboxylase	−	−	−	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	+	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	−	−	−
Hydrolysis of aesculin	+	+	−	+	+	+	+	NA	NA	−
Hydrolysis of gelatine	−	−	−	−	−	−	−	−	−	−
Arginine dihydrolase	−	−	+	−	−	−	−	+	−	−
Citrate utilization	−	−	+	−	−	−	−	−	−	+
Hydrolysis starch	−	−	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+
H2S production from cysteine	+	−	−	+	−	−	−	−	−	−
H2S production from peptone	+	−	−	+	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−	−	−	−	−
Fluorescent production on King B	−	−	−	−	−	−	−	−	−	−
Acid production from:										
Salicin	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	NA	+
D-fructose	+	+	+	+	+	+	+	+	+	+
D-saccharose	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+
Adonitol	−	−	−	−	−	−	−	−	−	NA
Trehalose	−	−	−	+	+	+	−	D	−	D
Amygdaline	−	−	−	+	+	−	−	+	−	−
Glycerol	+	+	+	+	+	+	−	+	+	+
Dulcitol	−	−	−	−	−	−	−	+	−	−
D-raffinose	−	+	−	+	−	+	−	+	+	−
Melibiose	+	−	−	+	−	+	−	+	+	−
D-xylose	−	−	+	−	−	−	−	+	−	−
D-galactose	−	−	+	+	+	−	−	+	+	D
D-sorbitol	−	−	+	+	−	+	−	+	+	−
L-arabinose	−	−	+	+	+	+	+	+	+	−
Cellobiose	+	−	V[f]	v	+	−	−	V	NA	+
Lactose	−	−	−	+	NA	NA	NA	−	−	−

[a] 1 = *Brenneria* sp. from Iran (*n* = 32), 2 = *Brenneria salicis* (*n* = 3), 3 = *Brenneria roseae* (*n* = 7), 4 = *Brenneria goodwinii* from Britain (*n* = 9), 5 = *Brenneria alni* (*n* = 5), 6 = *Brenneria nigrifluens* (*n* = 5), 7 = *Brenneria rubrifaciens* (*n* = 5), 8 = *Brenneria populi* subsp. *populi* from China (*n* = 5), 9 = *Brenneria populi* subsp. *brevivirga* (*n* = 2) from China, 10 = *Lonsdalea populi* (*n* = 5). *n*, Number of strains. Data for species 2, 4, 5, 6 and 7 taken from Denman, Brady (18); data for species 3 taken from Brady, Hunter (19); data for species 8 and 9 taken from Li, Fang (40) and Zheng, Piao (41); and data for species 10 taken from Li, Xue (42).
[b] −, negative, [c] +, 90−100% strains positive, [d] NA, not available., [e] D, 11−89% strains negative, [f] V, variable reaction.

Table 4. Isolates obtained from non-symptomatic *Populus nigra* trees sampled in this study

Preliminary identification	Isolate number	Tree location	Tree reference number
Gram-positive	P1-1	Hamadan, Bu Ali Sina University	20200001
Pantoea sp.	P1-2	Hamadan, Bu Ali Sina University	20200001
Pseudomonas sp.	P1-3	Hamadan, Bu Ali Sina University	20200001
Gram-positive	P1-4	Hamadan, Bu Ali Sina University	20200001
Pseudomonas sp.	P1-5	Hamadan, Bu Ali Sina University	20200001
Gram-negative	P1-6	Hamadan, Bu Ali Sina University	20200001
Gram-positive	P1-7	Hamadan, Bu Ali Sina University	20200001
Enterobacteriaceae	P1-8	Hamadan, Bu Ali Sina University	20200001
Gram-positive	P1-9	Hamadan, Bu Ali Sina University	20200001
Pseudomonas sp.	P2-1	Hamadan, Ganjnameh	20200002
Pseudomonas sp.	P2-2	Hamadan, Ganjnameh	20200002
Gram-positive	P2-3	Hamadan, Ganjnameh	20200002
Enterobacteriaceae	P2-4	Hamadan, Ganjnameh	20200002
Gram-positive	P2-5	Hamadan, Ganjnameh	20200002

MLSA

The nucleotide sequences of housekeeping genes were obtained for the representative isolates. PCR amplification and sequencing of the housekeeping

Fig. 3. BOX-PCR patterns of *Brenneria* causal agent of bark canker on poplar trees in Iran.

gene of the strains YMAPO5, YMAPO10, and YMAPO25 yielded partial sequences. Pairwise alignment revealed 100% sequence similarity between strains for all three housekeeping genes (*gyrB*, *infB*, and *rpoB*). They were 97.99, 99.35, and 97.96% identical with those of reference strain *B. salicis* LMG 2698 for *gyrB*, *infB*, and *rpoB* respectively. To compare poplar isolates with *Brenneria* strain NCPPB 4296, NCPPB 4297, and NCPPB 4299, no sequence of *gyrB*, *infB* and *rpoB* genes was available in GenBank. InMLSA based on the nucleotide sequences of the three housekeeping genes (*gyrB*, *infB*, and *rpoB*), the poplar isolates formed a separate branch from *B. salicis* strain LMG 2698 with 100% bootstrap in neighbour-joining (Fig. 5).
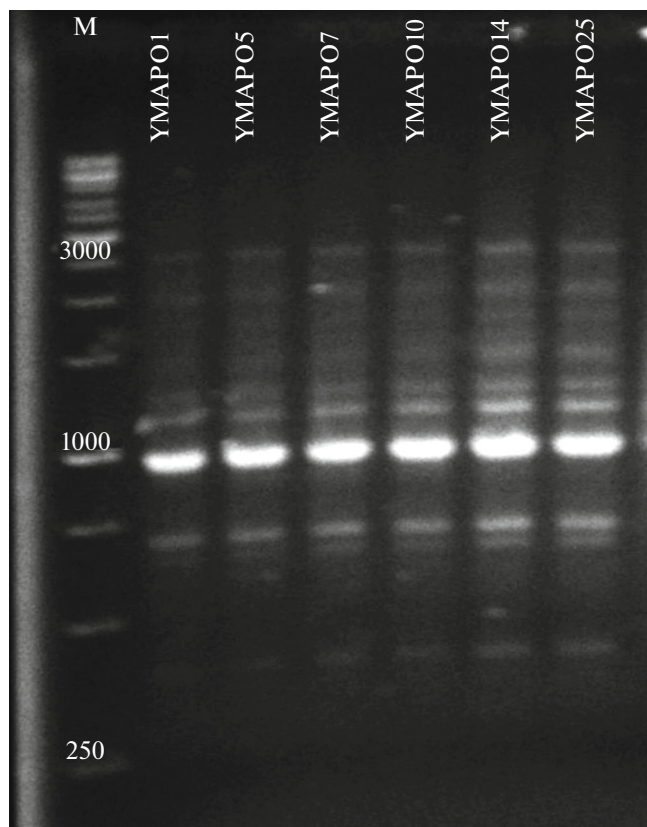
gapA Sequences Analysis

The representative isolates (YMAPO5, YMAPO10, and YMAPO25) demonstrated 100% similarity to each other in pairwise comparisons of the gapA gene sequences and 100 and 96.32% to NCPPB 4299, and *B. salicis* LMG 2698, respectively. In a phylogenetic tree based on the nucleotide sequences of *gapA* gene, poplar isolates clustered with those strains of *Brenneria* strain NCPPB 4296, NCPPB 4297, and NCPPB 4299 Separated from other species with 100% bootstrap in neighbour-joining (Fig. 6).

Pathogenicity Tests

Pathogenicity test 1: In the five days test period, all isolates obtained from symptomatic trees caused symptoms of bleeding canker on the_inoculated 30-cm-long stems. The outer and inner bark were macerated at the site of inoculation 2 days after inoc-
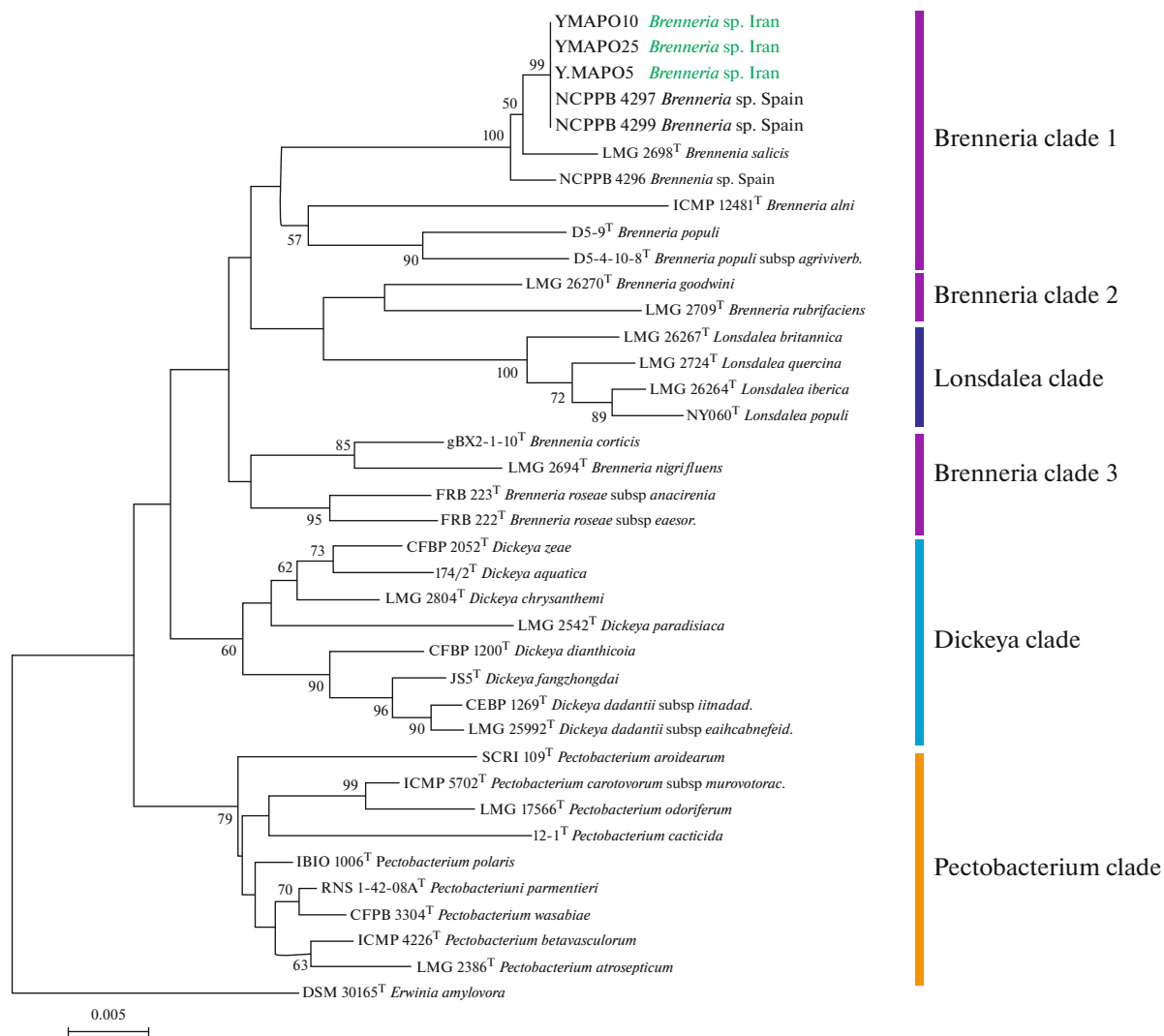
Fig. 4. The optimal neighbour-joining phylogenetic tree based on 16S rRNA sequences of poplar isolates (YMAPO5, YMAPO10, and YMAPO25) and the reference strains of *Brenneria*, *Lonsdalea Dickeya*, and *Pectobacterium* species obtained from GenBank (sum of branch length = 0.35196674). The GenBank accession numbers are given in parentheses behind the species names and strain numbers. Numbers at the nodes are the bootstrap values obtained for 1000 replicates. The sequence alignment consisted of 1263 characters. Bar, 0.005 substitution per nucleotide position. The tree is rooted to *Erwinia amylovora* LMG 30165T.

ulation, and a transparent yellowish liquid was exudated 5 days after inoculation (Fig. 1e).

Pathogenicity test 2: Fig. 1g shows the symptoms caused by isolates inoculated into excised stems. In the 4 days test period, the bark was macerated and after removing the bark, sapwood was necrotic at the site of inoculation; also. A transparent liquid flowed from the inoculation site. **Pathogenicity test 3:** inoculated isolates of symptomatic trees caused necrosis on shoots of poplar seedlings in greenhouse after 5 days inoculation (Fig. 1i).

In the Pathogenicity tests 1, 2, and 3, the inoculated stems with the bacterial isolates obtained from healthy trees showed no symptoms after 14 days. Therefore, they were not used in the pathogenicity test 4.

Pathogenicity test 4: typical field symptoms were reproduced including bleeding cankers with brownish exudates by symptomatic poplar isolates inoculated into mature *P. nigra* trees one month after inoculation (Fig. 1j). Re-isolations from the inoculated plants were done, and the isolated strains were confirmed to be *Brenneria* based on morphological characteristics and biochemical tests. No bacteria were re-isolated from the control plants. Inoculation with *B. goodwinii* ICMP21594 (Fig. and the control (SDW) did not cause any symptoms.
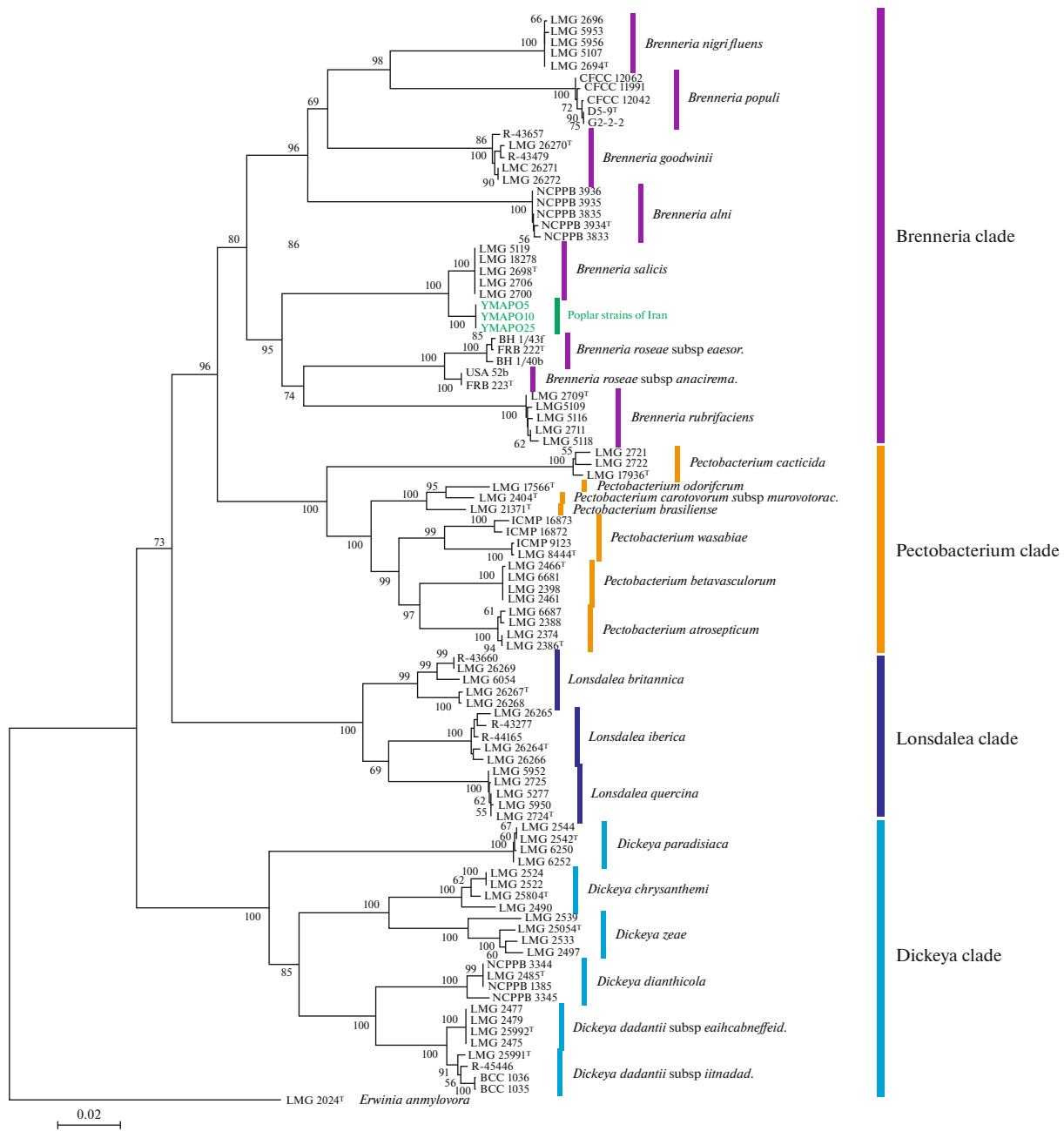
Fig. 5. The optimal neighbour-joining phylogenetic tree based on based on concatenated *gyrB*, *infB* and *rpoB* sequences of poplar isolates (YMAPO5, YMAPO10, and YMAPO25) and the reference isolates of *Brenneria*, *Lonsdalea Dickeya*, and *Pectobacterium* species obtained from GenBank (sum of branch length = 1.61974421). The GenBank accession numbers are given in parentheses behind the species names and strain numbers. Numbers at the nodes are the bootstrap values obtained for 1000 replicates. The sequence alignment consisted of 1868 characters. Bar, 0.02 substitution per nucleotide position. The tree is rooted to Erwinia amylovora LMG 2024.

DISCUSSION

During 2017 and 2018, sampling was done from poplar trees in different poplar plantations of Hamadan province, Iran. The disease was observed only on mature trees. The external symptoms were bleeding cankers with abundant dark brownish and slimy exudations which stained the trunk bark. The symptoms were similar to those caused by *Brenneria* spp. on the trunk of walnut, alder, poplar, and oaks trees [4, 11, 12, 14, 20]. When the outer bark was removedfrom the canker, the inner bark and sapwood showed a reddish color progressing to necrotic lesions. In Hungary [6] and China [7], the bark is vertically cracked in symptomatic trees, and a sticky, brown-colored fluid exudates from the canker. In Spain, two types of symp-
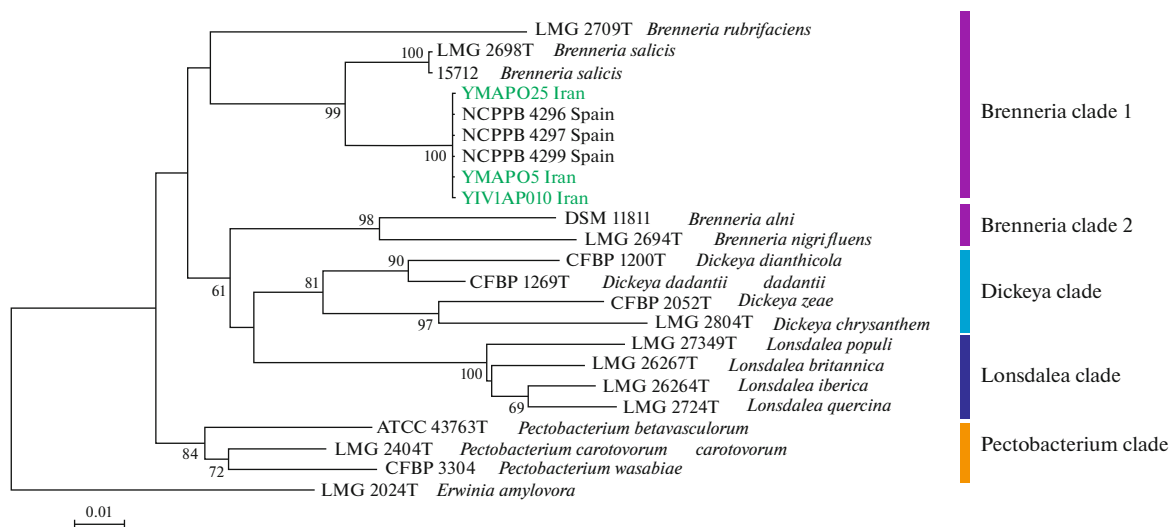
Fig. 6. The optimal neighbour-joining phylogenetic tree based on based on *gapA* sequences of poplar isolates (YMAPO5, YMAPO10, and YMAPO25) and the reference isolates of *Brenneria*, *Lonsdalea Dickeya*, and *Pectobacterium* species obtained from GenBank (sum of branch length = 0.75672445). The GenBank accession numbers are given in parentheses behind the species names and strain numbers. Numbers at the nodes are the bootstrap values obtained for 1000 replicates. The sequence alignment consisted of 397 characters. Bar, 0.01 substitution per nucleotide position. The tree is rooted to *Erwinia amylovora* LMG 2024.

toms were observed on poplar trees: [1] the bark canker is characterized by cankers with copious brownish exudations staining the trunk bark and reddish inner lesions progressing to necrosis that was caused by *Brenneria* sp. strain NCPPB 4296, NCPPB 4297, and NCPPB 4299 [4]; (2) the bark of symptomatic trees was vertically cracked and copious white frothy fluid and creamy slime was observed caused by *Lonsdalea populi* [5]. The symptoms observed in Iran were similar to bark canker of poplar trees caused by *Brenneria* sp. in Spain previously described by Biosca et al. [4]. None of the bacterial strains isolated from healthy trees were *Brenneria* spp. which suggests that they do not occur as endophytes. In addition, no fungi were isolated from diseased tissue. Thus the *Brenneria* sp. identified in this study from diseased trees is the causal agents of bleeding cankers on poplar trees in Iran.

Uniform colonies were always isolated from all the symptomatic trees (Fig. 2a). The repeated isolation of *Brenneria* sp. from necrotic tissues in diseased trees confirmed the etiology of this symptoms in poplar plantations in Iran. All of the poplar isolates obtained in this study were placed in one group based on phenotypic characteristics (Table 3) and BOX-PCR (Fig. 3). Several molecular methods are used to rapidly identify and classify pathogenic bacteria including BOX-PCR. There are three families of repetitive sequences for distinguish bacterial strains below the level of species including repetitive exteragenic palindromic (REP) sequence, enterobacterial repetitive intergenic consensus (ERIC) sequence and BOX element [28]. Previous studies have shown a high correlation between BOX-PCR fingerprinting and DNA-DNA homology

data [38]. The pathogenicity tests on excised stems and trunk of poplar trees reproduced the bark cankers observed in naturally infected poplar. The inoculations on shoots of poplar trees caused necrosis on them which was consistent with the Biosca et al. [4].

The poplar isolates from Iran were identified in this study as a previously undescribed *Brenneria* sp. Based on phenotypic and molecular characteristics poplar isolates were clustered with *Brenneria* sp. strains NCPPB 4296, 4297, and 4299 and are most closely related to *Brenneria salicis* based on 16S rRNA gene analysis (Fig. 4). They formed a separate cluster close to *B. salicis* based on MLSA analysis that this confirmed their *undescribed status* (Fig. 5). Nevertheless, the poplar isolates were differentiated phenotypically from their closest phylogenetic neighbours. MLSA is a useful tool for the investigation of bacterial taxonomic relationships [31, 32, 39] and an appropriate technique for species delineation and for distinguishing relationships at the intraspecific level. Because housekeeping genes are less conserved than rRNA genes, the high degree of sequence divergence of housekeeping genes is superior for identification purposes. MLSA was recently used with great success to resolve the phylogenetic position Brenneria species based on partial sequencing of *gyrB*, *rpoB*, *infB* and *atpD* genes [39]. The phylogenetic trees revealed that the strains isolated from bark canker on *P. nigra* in Iran (YMAPO5, YMAPO10, ad YMAPO25) and *Populus = eurameri-cana* in Spain (NCPPB 4296, 4297, and 4299) clustered together and close to the type strain of *B. salicis* LMG2698. The Poplar isolates were different in some phenotypic tests with those references strains of the

Brenneria species including *Brenneria salicis*. Unfortunately, No information has been published on phenotype characteristics of NCPPB 4296, 4297, and 4299. The results of phylogenetic analysis suggest that the bacterial strains obtained from poplar canker in Iran is similar to *Brenneria* sp. NCPPB 4296, 4297, and 4299 obtained from poplar canker in Spain [7]. Despite the similarities and differences, it cannot be stated that *Brenneria* sp. strains from Iran are a new species of Brenneria or a subspecies of *Brenneria salicis*. Whole genome sequencing (WGS) would need to be undertaken in order to formally describe this species.

Populus nigra is one of the most widely grown poplar cultivars in Iran. The bacterial canker disease observed to occur in Hamadan province of Iran. The occurrence of the bacterial canker disease on *P. nigra* emerged as a barrier for expanding cultivation of the poplar trees. In this study a previously undescribed *Brenneria* sp. was identified as causal agent bark canker of *P. nigra* in Iran and to our knowledge, this is the first report of the occurrence of this bacterial species in Iran, and on this host species globally.

ACKNOWLEDGMENTS

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Alimohamadi, A., Asadi, F., and Aghdaei, R.T., Genetic diversity in *Populus nigra* plantations from west of Iran, *Ann. For. Res.*, 2012, vol. 55, no. 1, pp. 165−178.

2. Arens, P., Coops, H., Jansen, J., et al., Molecular genetic analysis of black poplar (*Populus nigra* L.) along Dutch rivers, *Mol. Ecol.*, 1998, vol. 7, no. 1, pp. 11−8. https://doi.org/10.1046/j.1365-294x.1998.00316.x

3. Koning, H., Bacterial canker of the Poplar, *Chron. Bot.*, 1938, vol. 4, no. 1, pp. 11−12.

4. Biosca, E.G., Martín, S., Zuriaga, P., et al., Characterization of *Brenneria* sp. from poplar cankers in Spain, in *Modern Multidisciplinary Applied Microbiology: Exploiting Microbes and their Interactions*, Mendez-Vilas, A., Ed., Weinheim: Wiley, 2006, pp. 385−389.

5. Berruete, I., Cambra, M., Collados, R., et al., First report of bark canker disease of poplar caused by *Lonsdalea quercina* subp. *populi* in Spain, *Plant Dis.*, 2016, vol. 100, no. 10, p. 2159. https://doi.org/10.1094/PDIS-03-16-0405-PDN

6. Tóth, T., Lakatos, T., and Koltay, A., *Lonsdalea quercina* subsp. *populi* subsp. nov., isolated from bark canker of poplar trees, *Int. J. Syst. Evol. Microbiol.*, 2013, vol. 63, no. 6, pp. 2309−2313. https://doi.org/10.1099/ijs.0.042911-0

7. Li, Y., He, W., Ren, F., et al., A canker disease of *Populus × euramericana* in China caused by *Lonsdalea quercina* subsp. *populi, Plant Dis.*, 2014, vol. 98, no. 3, pp. 368−378.

8. Ridé, M., *Our Present Knowledge of Bacterial Canker on Poplar Caused by Aplanobacterium populi*, Rome: UN Food Agric. Org., 1963.

9. Hauben, L., Moore, E.R., Vauterin, L., et al., Phylogenetic position of phytopathogens within the Enterobacteriaceae, *Syst. Appl. Microbiol.*, 1998, vol. 21, no. 3, pp. 384−397. https://doi.org/10.1016/S0723-2020(98)80048-9

10. González, R., López-López, M., Biosca, E., et al., First report of bacterial deep bark canker of walnut caused by *Brenneria* (*Erwinia*) *rubrifaciens* in Europe, *Plant Dis.*, 2002, vol. 86, no. 6, p. 696.

11. Loreti, S., De Simone, D., and Gallelli, A., Detection and identification of *Brenneria nigrifluens*, the causal agent of the shallow bark canker of walnut by, PCR amplification, *J. Phytopathol.*, 2008, vol. 156, nos. 7−8, pp. 464−469. https://doi.org/10.1111/j.1439- 0434.2007.01393.x

12. Denman, S., Doonan, J., Ransom-Jones, E., et al., Microbiome and infectivity studies reveal complex polyspecies tree disease in Acute Oak Decline, *ISME J.*, 2018, vol. 12, no. 2, pp. 386−399. https://doi.org/10.1038/ismej.2017.170

13. McClean, A.E., Sudarshana, P., and Kluepfel, D.A., Enhanced detection and isolation of the walnut pathogen *Brenneria rubrifaciens*, causal agent of deep bark canker, *Eur. J. Plant Pathol.*, 2008, vol. 122, no. 3, pp. 413−424. https://doi.org/10.1007/s10658-008-9308-z

14. Surico, G., Mugnai, L., and Pastorelli, R., *Erwinia alni*, a new species causing bark cankers of alder (*Alnus Miller*) species, *Int. J. Syst. Evol. Microbiol.*, 1996, vol. 46, no. 3, pp. 720−726. https://doi.org/10.1099/00207713-46-3-720

15. Hauben, L., Steenackers, M., and Swings, J., PCR-based detection of the causal agent of watermark disease in willows (*Salix* spp.), *Appl. Environ. Microbiol.*, 1998, vol. 64, no. 10, pp. 3966−3971. https://doi.org/10.1128/AEM.64.10.3966-3971.1998

16. Sakamoto, Y., Occurrence of watermark disease of willows in Japan, *Plant Pathol.*, 1999, vol. 48, no. 5, pp. 613−619. https://doi.org/10.1046/j.1365-3059.1999.00368.x

17. Maes, M., Baeyen, S., De Croo, H., et al., Monitoring of endophytic *Brenneria salicis* in willow and its relation to watermark disease, in *Proc. 6th Plant Protection Sci. Conf.*, Prague, 2002, vol. 38, no. 2, pp. 528−530.

18. Denman, S., Brady, C., Kirk, S., et al., *Brenneria goodwinii* sp. nov., associated with acute oak decline in the UK, *Int. J. Syst. Evol. Microbiol.*, 2012, vol. 62, no. 10, pp. 2451−2456. https://doi.org/10.1099/ijs.0.037879-0

19. Brady, C., Hunter, G., Kirk, S., et al., Description of *Brenneria roseae* sp. nov. and two subspecies, *Brenneria roseae* subspecies *roseae* ssp. nov and *Brenneria roseae* subspecies *americana* ssp. nov. isolated from symptomatic oak, *Syst. Appl. Microbiol.*, 2014, vol. 37, no. 6, pp. 396−401. https://doi.org/10.1016/j.syapm.2014.04.005

20. Moradi-Amirabad, Y., Rahimian, H., and Babaeizad, V., *Brenneria* spp. and *Rahnella victoriana* associated with acute oak decline symptoms on oak and hornbeam in Iran, *Forest Pathol.*, 2019, vol. 49, p. e12535. https://doi.org/10.1111/efp.12535

21. *Laboratory Guide for the Identification of Plant Pathogenic Bacteria*, Schaad, N.W., Jones, J.B., and Chun, W., Eds., St. Paul: APS Press, 2001.

22. Suslow, T., Schroth, M., and Isaka, M., Application of a rapid method for Gram differentiation of plant pathogenic and saprophytic bacteria without staining, *Phytopathology*, 1982, vol. 72, no. 7, pp. 917−918. https://doi.org/10.1094%2FPhyto-72-917

23. Hugh, R. and Leifson, E., The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria, *J. Bacteriol.*, 1953, vol. 66, no. 1, pp. 24−26.

24. Kovacs, N., Identification of *Pseudomonas pyocyanea* by the oxidase reaction, *Nature*, 1956, vol. 178, no. 4535, p. 703. https://doi.org/10.1038/178703a0

25. Ayers, S.H., Rupp, P., and Johnson, W.T., *A study of the Alkali-Forming Bacteria Found in Milk*, Washington, DC: US Department of Agriculture, 1919.

26. Moradi Amirabad, Y. and Khodakaramian, G., Isolation and characterization of *Erwinia pirifloriniigrans* causal agent flower necrosis of red poppy, *Australas. Plant Pathol.*, 2017, vol. 46, no. 6, pp. 611−616. https://doi.org/10.1007/s13313-017-0513-0

27. *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Ausubel, F.M., Ed., New York: Wiley, 2002, 5th ed.

28. Versalovic, J., Schneider, M., De Bruijn, F., et al., Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction, *Methods Mol. Cell. Biol.*, 1994, vol. 5, no. 1, pp. 25−40.

29. Charkhabi, N.F., Shams-bakhsh, M., and Rahimian, H., Genetic diversity among *Brenneria nigrifluens* strains in Iran, *Eur. J. Plant Pathol.*, 2010, vol. 128, pp. 303−310. https://doi.org/10.1007/s10658-010-9667-0

30. Weisburg, W.G., Barns, S.M., Pelletier, D.A., et al., 16S ribosomal DNA amplification for phylogenetic study, *J. Bacteriol.*, 1991, vol. 173, no. 2, pp. 697−703. https://doi.org/10.1128/jb.173.2.697-703.1991

31. Brady, C., Cleenwerck, I., Venter, S., et al., Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA), *Syst. Appl. Microbiol.*, 2008, vol. 31, no. 6, pp. 447−460. https://doi.org/10.1016/j.syapm.2008.09.004

32. Ma, B., Hibbing, M.E., Kim, H.S., Reedy, R.M., et al., Host range and molecular phylogenies of the soft rot enterobacterial genera *Pectobacterium* and *Dickeya*, *Phytopathology*, 2007, vol. 97, no. 9, pp. 1150−1163. https://doi.org/10.1094/PHYTO-97-9-1150

33. Hall, T.A., BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucleic Acids Symp. Ser.*, 1999, vol. 41, pp. 95−98.

34. Altschul, S.F., Gish, W., Miller, W., et al., Basic local alignment search tool, *J. Mol. Biol.*, 1990, vol. 215, no. 3, pp. 403−410. https://doi.org/10.1016/S0022-2836(05)80360-2

35. Thompson, J.D., Higgins, D.G., and Gibson, T.J., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res.*, 1994, vol. 22, no. 22, pp. 4673−4680. https://doi.org/10.1093/nar/22.22.4673

36. Kumar, S., Stecher, G., Li, M., et al., MEGA X: molecular evolutionary genetics analysis across computing platforms, *Mol. Biol. Evol.*, 2018, vol. 35, no. 6, pp. 1547−1549. https://doi.org/10.1093/molbev/msy096

37. Felsenstein, J., Confidence limits on phylogenies: an approach using the bootstrap, *Evolution*, 1985, vol. 39, no. 4, pp. 783−791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

38. Lanoot, B., Vancanneyt, M., Dawyndt, P., et al., BOX-PCR fingerprinting as a powerful tool to reveal synonymous names in the genus *Streptomyces*. Emended descriptions are proposed for the species *Streptomyces cinereorectus, S. fradiae, S. tricolor, S. colombiensis, S. filamentosus, S. vinaceus* and *S. phaeopurpureus, Syst. Appl. Microbiol.*, 2004, vol. 27, no. 1, pp. 84−92.

39. Brady, C.L., Cleenwerck, I., Denman, S., et al., 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, Int. J. Syst. Evol. Microbiol.*, 2012, vol. 62, no. 7, pp. 1592−602. https://doi.org/10.1099/ijs.0.035055-0

40. Li, Y., Fang, W., Xue, H., Liang, W.X., et al., *Brenneria populi* sp. nov., isolated from symptomatic bark of *Populus* × *euramericana* canker, *Int. J. Syst. Evol. Microbiol.*, 2015, vol. 65, no. 2, 432−437. https://doi.org/10.1099/ijs.0.066068-0

41. Zheng, M.H., Piao, C.G., Xue, H., et al., *Brenneria populi* subsp. *brevivirga* subsp. nov. isolated from symptomatic bark of *Populus* × *euramericana* canker, and description of *Brenneria populi* subsp. *populi* subsp. nov., *Int. J. Syst. Evol. Microbiol.*, 2017, vol. 67, no. 9, pp. 3633−3638. https://doi.org/10.1099/ijsem.0.002183

42. Li, Y., Xue, H., Guo, L.M., et al., Elevation of three subspecies of *Lonsdalea quercina* to species level: *Lonsdalea britannica* sp. nov., *Lonsdalea iberica* sp. nov. and *Lonsdalea populi* sp. nov., *Int. J. Syst. Evol. Microbiol.*, 2017, vol. 67, no. 11, pp. 4680−4684. https://doi.org/10.1099/ijsem.0.002353