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Bacterial Canker Disease on *Populus* × *euramericana* Caused by *Lonsdalea populi* in Serbia

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Abstract: *Populus* × *euramericana* (Dode) Guinier clone (cl.) "I-214" is a fast-growing interspecific hybrid between Eastern cottonwood (*P. deltoides* Bartr. ex Marsh) and European black poplar (*Populus nigra* L.). *Populus* × *euramericana* was introduced into Serbia in the 1950s and has become one of the most widely grown poplar species. In September 2019, cankers were observed on stems and branches of *P.* × *euramericana* cl. "I-214" trees in a two-year-old poplar plantation in the province of Vojvodina, Serbia. The canker tissue was soft and watery, and a colorless fluid that smelled rotten flowed from the cracks in the bark, suggesting possible bacterial disease. After two weeks, diseased trees experienced crown die-back and oozing of foamy, odorous exudates and this study aimed to identify the causal agent of the disease. Canker margins and exudates were collected from 20 symptomatic trees. The associated bacterium was isolated and identified using biochemical characteristics, phylogenetic analyses based on 16S rRNA gene sequences, and multilocus sequence analyses (MLSA) based on partial sequencing of three housekeeping genes (*gyrB*, *infB*, and *atpD*). The pathogen was identified as *Lonsdalea populi*. Pathogenicity tests were conducted on rooted cuttings of *P.* × *euramericana* cl. "I-214" in an environmental test chamber and demonstrated that the isolated bacterial strain was able to reproduce symptoms of softened, water-soaked cankers and exudation. To the best of our knowledge, this is the first report of *L. populi* causing bacterial canker disease on *P.* × *euramericana* cl. "I-214" in Serbia and in southeastern Europe (SEE). It is also the first report of a bacterial disease on hybrid poplars, including *P.* × *euramericana* in this country and in SEE. If the disease spreads into new areas, selection for *L. populi* resistance may need to be integrated into future poplar breeding programs.

Keywords: *Populus* × *euramericana*; *Lonsdalea populi*; canker diseases; poplar diseases; bacterial canker of poplars; die-back of poplars; MLSA

1. Introduction

Canadian poplar (*Populus* × *euramericana* (Dode) Guinier, syn. *Populus* × *canadensis* Moench) is a fast-growing interspecific hybrid between North American Eastern cottonwood (*Populus deltoides* Bartr. ex Marsh ♀) and European black poplar (*Populus nigra* L. ♂). It is an important tree species in many European countries. *Populus* × *euramericana* is characterized by rapid growth rates, ease of clonal propagation, coppice regeneration, high biomass production and carbon sequestration, potential

for phytoremediation, and suitability for multiple industrial uses, e.g., sawn timber, veneers, and fuelwood [1–3].

In Serbia, *P.* × *euramericana* is the most widely grown poplar species [4]. It is cultivated on floodplains and along the riverbanks of the major Serbian lowland rivers, i.e., the Danube, Sava, Tisa, Tamiš, and Morava on hydromorphic soil types, including fluvisol, humofluvisol and humogley [5]. Although several clones and cultivars of *P.* × *euramericana* are used in Serbia, *P.* × *euramericana* clone (cl.) "I-214" is the most common, most productive, and the most economically important poplar clone in the country [4,6].

Several fungal diseases are known to affect *P.* × *euramericana* cl. "I-214" in Europe. These include *Dothichiza* canker caused by *Dothichiza populea*, Sacc. et Briard, *Marssonina* leaf spot caused by *Drepanopeziza brunnea* (Ellis & Everh.) Rossman & W.C. Allen, *Cytospora* canker caused by *Cytospora chrysosperma* (Pers.) Fr., *Botryosphaeria* canker caused by *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. et De Not., and *Melampsora* leaf rust caused by *Melampsora* spp. Moreover, *Xanthomonas populi* (ex-Ridé 1958) Ridé and Ridé 1992 and *Lonsdalea populi* (Tóth et al. 2013) Li et al. 2017 have been reported as causal agents of bacterial canker disease on *P.* × *euramericana* in poplar plantations [7–9].

Populus × *euramericana* cl. "I-214" was introduced into Serbia in the 1950s. At the time of introduction this clone was shown to be highly productive and resistant to various diseases, including spring defoliation caused by *Venturia populina* (Vuill.) Fabric., *Dothichiza* canker, leaf curl caused by *Taphrina populina* Fr. (Fr.), *Melampsora* leaf rust and mosaic virus disease caused by poplar mosaic virus (PMV) [4,9]. However, during the past 70 years, *P.* × *euramericana* cl. "I-214" has gradually become susceptible to multiple leaf and stem diseases, i.e., *Marssonina* leaf spot, *Venturia* spring defoliation, *Melampsora* leaf rust, and *Dothichiza* and *Cytospora* stem canker [9].

In September 2019, symptoms of a bacterial canker disease were observed in a two-year-old *P.* × *euramericana* cl. "I-214" plantation in Vojvodina, Serbia. Affected trees initially exhibited longitudinal cracks in the bark of the stems and branches accompanied by oozing of a small amount of colorless or whitish sap. As the disease progressed, the cracks in the bark enlarged, the vascular tissues under the bark became necrotic, soft, and water-soaked and copious amounts of sticky and often foamy sap with a rotten smell flowed from the cracks. Once exposed to the air the sap gradually darkened, becoming reddish or brownish and causing staining of the tree bark (Figure 1a–d). In some cases, the infected bark peeled away from the sunken canker area exposing a creamy mass of whitish exudates with a fermentation odor and these cankers usually appeared on the bark surface of the lower trunk. In severe cases of the disease, cankers caused crown die-back and the diseased trees died within a few weeks (Figure 1d). These symptoms resembled those of a recently described bacterial canker disease of hybrid poplars in Hungary, Portugal, Spain, and China caused by *L. populi* [8,10–12]. The aim of this study was to identify the bacterium associated with the disease symptoms observed on *P.* × *euramericana* cl. "I-214" trees in Serbia. This was done using biochemical characteristics, phylogenetic analyses based on 16S ribosomal RNA (rRNA), multilocus sequence analyses (MLSA) of three housekeeping genes, i.e., part of the DNA gyrase subunit B (*gyrB*), translation initiation factor IF2 (*infB*) and ATP synthase subunit beta (*atpD*), and a pathogenicity test.

Figure 1. Bacterial canker disease on *Populus* × *euramericana* clone "I-214" caused by *Lonsdalea populi* in Serbia. (**a**) Necrotic bark with foamy sap flowing from the infection site. (**b**) Canker with cracked bark and exudates emerging from the infected stem. (**c**) Softening and darkening of the vascular part of the trunk in the cankered area. (**d**) Dead tree with exudates staining the bark. (**e**) Colonies of *L. populi* after 24 h of incubation at 30 °C on tryptone soya agar. (**f**) Water-soaked sunken canker formed on *P.* × *euramericana* rooted cutting one month after inoculation with *L. populi*. (**g**) Dark, soft, and watery wood beneath the bark of a canker formed on *P.* × *euramericana* rooted cutting one month after inoculation with *L. populi*. (**h**) Negative control showing absence of canker development.

2. Materials and Methods

2.1. Sample Collection, Isolation, and Biochemical Characterization

For pathogen isolation, stem and branch tissues showing symptoms of a bacterial canker and whitish creamy exudates were collected using sterile equipment from twenty symptomatic *P.* × *euramericana* cl. "I-214" trees grown in a poplar plantation near Glogonj, in Vojvodina, Serbia (N 44°59′; E 20°32′). Samples of cankers and exudates were placed in polyethylene bags and sterile 2 mL

Eppendorf tubes, respectively, and kept at 4 °C until isolation was undertaken. Small pieces (3–5 mm diameter) of woody tissue were cut from the canker margins, surface sterilized using 70% (v/v) ethanol for 1 min. followed by a solution of 10% (v/v) sodium hypochlorite for 1 min., and then rinsed in sterile distilled water. The tissue was macerated in 1 mL of sterile distilled water using a sterile mortar and pestle; the resulting suspension was transferred to 2 mL Eppendorf tubes, and shaken for 2 min. using ZX3 advanced vortex mixer (VELP Scientifica, Milan, Italy). The suspension was serially diluted (10-fold dilutions to 10^{-9}) and 50 µL of each dilution was spread onto tryptone soya agar (TSA, Titan Biotech Ltd., New Delhi, India). Moreover, samples of exudates were diluted in the same manner and spread onto TSA. Petri dishes were incubated at 30 °C for 48 h (Heidolph incubator 1000, Heidolph co., Kelheim, Germany). In total, seven bacterial colony types were isolated. The prevalent colonies were similar in appearance to *L. populi*, i.e., white-ivory colored, slightly bluish on the underside, round, and slightly convex [8]. These colonies were purified by subculturing and subjected to Gram test which was performed using a non-staining method with a 3% KOH solution [13]. Gram negative bacterial strains were further examined with an Olympus BX53F light microscope (Olympus Co., Tokyo, Japan) at ×400 and ×1000 magnification using an Olympus SC50 digital camera and accompanying software. A Gram-negative strain with cell morphology like *L. populi* (cells 0.5–1 × 1–2 µm in size, short-rod-shaped, motile, occurring single, or aggregated in clumps, Figure S1) [8] was selected and named ILFE-LP1. The strain was stored in tryptone soya broth (TSB, Titan Biotech Ltd., New Delhi, India) containing 40% glycerol (v/v) at −80 °C, deposited in the culture collections of the Institute of Lowland Forestry and Environment (ILFE) and NARIC Fruitculture Research Institute and further used in this study to identify the bacterium and confirm its pathogenicity.

The analyses of biochemical characteristics of the bacterium were conducted using API 20E kit (Bio-Mérieux, Marcy L'Etoile, France) following the manufacturer's instructions and the test strip was incubated for 24 h. A type strain of *L. populi* (NY060, provided by the NARIC Forest Research Institute, Mátrafüred, Hungary) was used as a positive control and isolates were assessed twice.

2.2. DNA Extraction, PCR Amplification, and Sequencing

Total bacterial genomic DNA was isolated from cells harvested from culture grown for 24 h at 30 °C in TSB using a mericon DNA bacteria kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The DNA was quantified with a nanodrop (BioSpec-nano, Shimadzu Biotech, Kyoto, Japan), stored at −20 °C and diluted to the concentration of 20 ng/µL prior to use in PCR reactions. Partial 16S rRNA gene was amplified by PCR using the primers and conditions as published by [14] (Table 1). Three housekeeping genes, including *gyrB*, *infB*, and *atpD* were amplified using primers designed by [15] (Table 1). The conditions for PCR amplification of the housekeeping genes were as previously determined by [16]. The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in 1 x TBE buffer, stained with Roti-GelStain (Carl Roth, Karlsruhe, Germany) and visualized under UV illumination. The size of the products was estimated using O'gene ruler 100bp DNA ladder (Thermo Fisher Scientific Inc., Bremen, Germany). The PCR products were purified using a PCR purification kit (QIAquick, Qiagen, Hilden, Germany). Sanger sequencing was performed by Mycrosynth (Balgach, Switzerland) using primers designed by [14,15] (Table 1).

2.3. Phylogenetic Analyses

Raw sequence data were examined and combined into a consensus sequence using BioEdit version 7.2.5 [17] and MEGA X [18]. Sequences were compared to those of the other *Lonsdalea* strains available in the GenBank database using BLAST and related sequences were downloaded and included in the analyses (Table S1). Multiple sequence alignments were obtained with MAFFT version 7 (on-line version) [19], checked manually for alignment errors in MEGA X [18] and corrected where necessary. The phylogenetic analyses were performed using Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses. ML analyses were conducted for both 16S rRNA and the combined data set of three housekeeping genes, whereas MP analyses were run only for the combined data set. The

MP analyses and the partition homogeneity test (PHT) were conducted as described by [20] and they were performed in PAUP version 4.0b10 [21]. ML analyses were run using an online version of PhyML 3.0 [22] by applying smart model selection [23]. Bootstrap analysis was carried out using 1000 replicates [24]. Phylogenetic trees were visualized using MEGA X [19]. The DNA sequences of isolate ILFE-LP1 obtained in this study were deposited in GenBank (MT505705-16S, MT537174- *atpD*, MT559754-*gyrB*, and MT559753-*infB*, Table S1).

Table 1. Primers used in this study to amplify and sequence 16S rRNA gene and housekeeping genes (*gyrB*, *atpD* and *infB*) from ILFE-LP1 bacterial strain isolated from a *Populus* × *euramericana* cl. "I-214" tree with symptoms of a bacterial canker in Serbia.

PCR Primers	Sequence	Reference
16SP1	5′-GAAGAGTTTGATCATGGCTC-3′	[15]
16SP2	5′-AAGGAGGTGATCCAGCCGCA-3′	[15]
gyrB 01-F	5′-TAARTTYGAYGAYAACTCYTAYAAAGT-3′	[16]
gyrB 02-R	5′-CMCCYTCCACCARGTAMAGTT-3′	[16]
atpD 01-F	5′-RTAATYGGMGCSGTRGTNGAYGT-3′	[16]
atpD 02-R	5′-TCATCCGCMGGWACRTAWAYNGCCTG-3′	[16]
infB 01-F	5′-ATYATGGGHCAYGTHGAYCA-3′	[16]
infB 02-R	5′-ACKGAGTARTAACGCAGATCCA-3′	[16]
Sequencing Primers		
SP1	5′-ACCGCGGCTGCTGGCACG-3′	[15]
SP2	5′-CTCGTTGCGGGACTTAAC-3′	[15]
16SP2	5′-AAGGAGGTGATCCAGCCGCA-3′	[15]
gyrB 07-F	5′-GTVCGTTTCTGGCCVAG-3′	[16]
gyrB 08-R	5′-CTTTACGRCGKGTCATWTCAC-3′	[16]
atpD 03-F	5′-TGCTGGAAGTKCAGCARCAG-3′	[16]
atpD 04-R	5′-CCMAGYARTGCGGATACTTC-3′	[16]
infB 03-F	5′-ACGGBATGATYACSTTCCTGG-3′	[16]
infB 04-R	5′-AGYTTAGATTTCTGCTGACG-3′	[16]

2.4. Pathogenicity Test

In January 2020, shoots were collected from stooled beds of *P.* × *euramericana* cl. "I-214" established at an experimental forest nursery "Kaćka forest" of ILFE in Kać, Novi Sad (N 45°17′; E 19°53′). Dormant cuttings (diameter: 14 ± 0.3 mm; length: 30 ± 0.2 cm) were prepared from the lower parts of the collected shoots and stored in polyethylene bags in a cold chamber at 4 °C for two months. In March 2020, the cuttings were first soaked in water for two days in the dark at room temperature (18 ± 2 °C). They were then surface sterilized using 70% ethanol (v/v) and their top ends were sealed using grafting wax (Savacoop, Novi Sad, Serbia) to prevent desiccation and contamination. The cuttings were placed in 3 L plastic pots containing loamy fluvisol soil [5] obtained from the "Kaćka forest" nursery. They were kept in a greenhouse at ILFE (23 ± 2 °C day temperature, 19 ± 2 °C night temperature, 60–70% humidity, 16/8h day/night cycle) for one month and watered every other day to field capacity.

Thirty cuttings that developed roots were transferred to an environmental test chamber (Sanyo, MLR-351H) for the pathogenicity test. They were arranged in a completely randomized design with ten replicates (poplar plants) per treatment. Plants were inoculated with Serbian strain ILFE-LP1 and a Hungarian type strain of *L. populi* NY060 to serve as a positive control. Negative controls were mock inoculated using sterile distilled water. Prior to inoculation, bacterial strains were cultured for 24 h

at 30 °C on TSA. Inoculum was prepared in 20 mL TSB. Single bacterial colonies were transferred to Erlenmeyer flasks and incubated at 30 °C and 180rpm for 24 h in a shaker incubator (Unimax 1010, Heidolph co., Kelheim, Germany). The number of colony forming units (CFU)/ml was determined by spread-plate technique [25] on TSA incubated at 30 °C for 24 h. Bacterial suspension in TSB was transferred to 2 mL Eppendorf tubes, centrifuged at 13,200 rpm for 10 min. and the inoculum concentration was adjusted with sterile distilled water to 10^8 CFU/ml. Plants were first surface sterilized using 70% ethanol (v/v) and then a cork borer was used to create a 6 mm diameter wound in the middle of each plant. The bacterial suspension was injected into the wound (40 µL) using a pipette and the wound was sealed with Parafilm (Pechiney, Chicago, IL, USA) to retain moisture and protect the wound from contamination. The inoculated plants were maintained at 28 °C, with a relative humidity of 90% under a 16/8 day/night cycle [26] and watered as described above. The experiment was carried out for one month and plants were monitored every day for the appearance of symptoms. At the end of the experiment, the presence of external and internal symptoms was recorded, and the length of internal canker lesions was measured. The pathogenicity test was repeated once.

2.5. Statistical Analyses

Statistical analyses of pathogenicity experiment data were performed using Statistica 12.0 (StatSoft Inc., Tulsa, OK, USA). The data were checked for normality using Kolmogorov−Smirnov test and homogeneity of variances was tested with Levene's test. The analyses were further conducted using non-parametric Mann−Whitney U test (α = 0,05). Because there were no significant differences in lesion lengths produced by the same strain in the two subsequent pathogenicity trials, the data from a single strain were combined for further analyses.

3. Results

3.1. Biochemical Characterization

Isolate ILFE-LP1 was positive for acetoin and citrate utilization, and negative for β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophan deaminase, indole, and gelatinase production. Acid was produced from D-glucose, D-mannitol, D-sucrose, and amygdalin. Nitrates were not reduced to nitrites. Biochemical characteristics of ILFE-LP1 resembled those of the type strain NY060 of *L. populi*.

3.2. Phylogenetic Analyses

The 16S data set contained 12 sequences and 1351 characters (1282 parsimony informative, 46 parsimony uninformative, CI = 0.9, RI = 0.8, TL = 76) and the model HKY85+I was chosen for the ML analyses (I = 0.897). The topology of the ML and MP phylogenetic trees was similar, and the ML tree is presented (Table S1, Figure S2).

The combined dataset of three housekeeping genes contained 36 sequences with *Brenneria nigrifluens* as an outgroup (Table S1). The sequence dataset contained 1833 characters (195 parsimony informative, 1638 parsimony uninformative, CI = 0.8, RI = 0.9, TL = 320). The result of the PHT test was not significant and showed that three loci can be combined (P = 0.03). The model GTR+G+I was chosen for the ML analyses (G = 0.507, I = 0.451). The MP and ML analyses produced phylogenetic trees with the similar topology and therefore, only the ML tree is shown (Figure 2). Serbian strain ILFE-LP1 formed a monophyletic clade with *L. populi* strains from Hungary, Portugal, and China within *Lonsdalea* species in the phylogenetic analyses. The separation of *L. populi* from other *Lonsdalea* species was moderately supported in the phylogenetic analyses of 16S rRNA sequences (bootstrap support = 85% ML, MP) and strongly supported in the phylogenetic analyses of a combined *atpD*, *gyrB* and *infB* dataset (bootstrap support = 81% ML, 100% MP). Although the branch lengths indicate differences in the concatenated sequences, the scale bar represents 0.001 nucleotide changes per site.

Based on phylogenetic analyses, strain ILFE-LP1 isolated in this study was identified as *L. populi* (Figure 1, Figure S2).

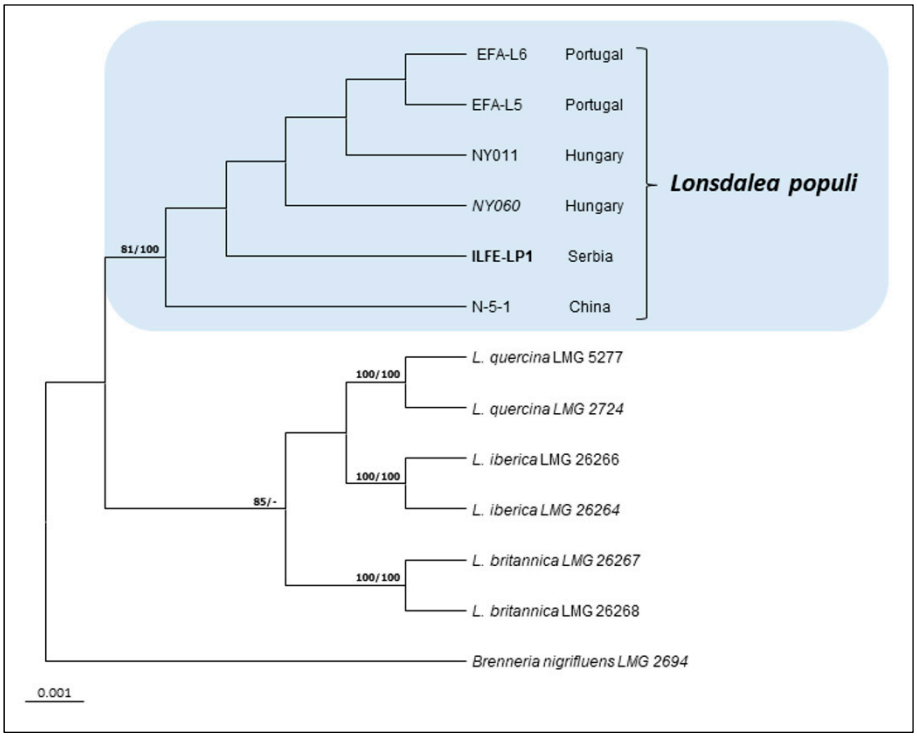

Figure 2. Maximum-likelihood (ML) tree resulting from ML analyses of the concatenated ***atpD***, ***gyrB*** and ***infB*** gene sequences and showing the phylogenetic position of *Lonsdalea populi* in relation to its closely related species. The bootstrap support values (ML/MP ≥ 80% (maximum parsimony: MP)) are indicated at the nodes, and the scale bar represents the expected number of changes per site. The tree was rooted to *Brenneria nigrifluens*. Strain ILFE-LP1 identified in this study is shown in bold and a clade corresponding to *L. populi* is highlighted.

3.3. Pathogenicity Test

Four days after inoculation, the oozing of a colorless fluid and a small sunken area around the inoculation site were evident on most stems inoculated with a bacterial suspension of ILFE-LP1 and on stems of a positive control inoculated with *L. populi* NY060. The lesion gradually expanded further in the following days and oozing continued. One month after inoculation, sunken, water-soaked external cankers were visible on each stem inoculated with isolates ILFE-LP1 and *L. populi* NY060 and after the bark was removed, water-soaked, necrotic lesions were observed around the inoculation points and measured from 1.4 to 3.6 mm (average length = 1.8 mm) and 1.4 to 3.8 mm (average length = 1.9 mm), respectively (Figure 1). There were no significant differences in lesion lengths between the two strains ($p = 0.47$). No cankers formed on the stems of the control plants. *Lonsdalea populi* was successfully (100%) re-isolated from canker margins on TSB and its identity was confirmed using morphology, Gram test, PCR, and sequencing of the *atpD* gene following the procedure described above (Figure S3). *Lonsdalea populi* was never isolated from negative controls.

4. Discussion

This study provides the first record of *L. populi* on *P.* × *euramericana* cl. "I-214" in Serbia, and southeastern Europe (SEE). The geographic range of this Gram-negative bacterium has extended and its host association with *P.* × *euramericana* was confirmed. *Lonsdalea populi* was identified using a polyphasic approach, i.e., biochemical characteristics, phylogenetic analyses of the 16S rRNA, and

MLSA of three housekeeping genes (*gyrB*, *infB* and *atpD*). The pathogenicity test confirmed that *L. populi* is the causal agent of the bacterial canker disease of *P.* × *euramericana* cl. "I-214" in Serbia.

The present study is also the first report of a bacterial disease on hybrid poplars, including *P.* × *euramericana* in Serbia, and SEE. Despite the importance of hybrid poplars, no previous research has been conducted on bacterial diseases of these trees in Serbia and SEE. Moreover, little research has been conducted on bacterial diseases of *Populus* spp. in SEE and only 'Candidatus Phytoplasma asteris'-related phytoplasmas (yellow disease phytoplasmas) were reported from Lombardy poplar (*Populus nigra* L. 'Italica') trees planted as ornamentals in Belgrade, Serbia and in Zagreb, Croatia [27,28].

The isolation of *L. populi* from *P.* × *euramericana* in Serbia is not surprising, given that it is a well-known pathogen that causes bacterial canker disease of hybrid poplars [12]. *Lonsdalea populi* has been isolated from *P.* × *euramericana* in previous studies in Hungary, Spain, Portugal, and China [8,10–12]. It has also been found associated with *P.* × *interamericana* in Spain and recently reported as a pathogen of Chinese willow (*Salix matsudana* Koidz.) causing cankers with large amounts of white sour exudates in China [11,29].

The disease symptoms (oozing cankers with water-soaked, soft wood) caused by *L. populi* observed in this study are consistent with previous reports of *Lonsdalea* canker of poplars [8,10–12]. In the current study, however, *L. populi* was isolated from two-year old *P.* × *euramericana* trees, whereas in other countries it was found on more than three-year-old trees [8,10–12]. Symptoms of a bacterial canker disease on *P.* × *euramericana* have previously also been reported associated with *Xanthomonas populi* (Ridé) Ridé and Ridé. This bacterium was a major concern in poplar-growing regions of Europe in the 1950s [30]. However, symptoms observed in this study were not typical of those caused by *X. populi* and swollen cankers with deep cracks in the bark have not been observed in the field. Moreover, *Neocosmospora solani* sensu lato has been found associated with cankers of hardwood trees, including *Populus* spp. [31]. Likewise, apart from *L. populi*, Li et al. [12] isolated *N. solani* (Mart.) L. Lombard & Crous from diseased tissues of *P.* × *euramericana* trees experiencing stem cankers in China. The authors, however, reported that *N. solani* is not an aggressive pathogen of this tree species and concluded that *L. populi* is the causal agent of a canker disease of *P.* × *euramericana* in China. Nevertheless, because disease symptoms indicated a possible bacterial infection, fungal isolations were not performed in this study, but additional research is currently being conducted to see if *L. populi* alone is causing the canker symptoms observed in the field.

To prevent the spread of the disease into new areas and plantations, in this study, a pathogenicity test was conducted using *P.* × *euramericana* rooted cuttings in an environmental test chamber under controlled conditions as described in Hou et al. [26]. Due to the high humidity to which poplars were exposed during the test (90%) to promote bacterial activity, and the fast-growing nature of *P.* × *euramericana* the experiment lasted for one month and symptoms of water-soaked cankers with exudation were successfully reproduced. Moreover, cankers of a similar size were formed when *P.* × *euramericana* was inoculated with the strain type of *L. populi* that was used as a positive control. However, oozing was not as abundant, foamy, and creamy as seen on trees in the field in an advanced stage of the disease development. This may be due to the age of the plants used for inoculation, and the duration of the test. Similarly, in a study of Li et al. [12] *L. populi* induced canker symptoms when inoculated into water-cultured excised stems in an environmental test chamber, but abundant, white, sour exudates were observed only when the test was carried out under field conditions using 3–5-year old trees. Difficulties in reproducing disease symptoms in pathogenicity trials have also been reported for other plant pathogenic bacteria, including *Lonsdalea quercina* (Hauben et al. 1999) Brady et al. 2012, *Brenneria nigrifluens* (Wilson et al. 1957) Hauben et al. 1999, and *Brenneria rubrifaciens* (Wilson et al. 1957) Hauben et al. 1999 [32–34].

The occurrence and pathogenicity of *L. populi* on *P.* × *euramericana* cl. "I-214" is of a major concern in Serbia because cl. "I-214" is the most widely grown and economically important poplar clone in the country. Intensively cultured plantations (even-aged, clonal stands) and monoclonality have already increased the vulnerability of this clone to various leaf and canker diseases [4,9]. Because the use

of antibiotics for plant disease control in Serbia is prohibited [35], management options for bacterial disease problems in Serbian poplar plantations are limited. Therefore, genetic improvement programs that continuously screen new clones for disease resistance while assuring highest possible volume production could be the most promising strategy to combat *Lonsdalea* canker of poplars. Moreover, an integrated approach of disease prevention and control, focusing not only on selection and breeding for resistance, but also on biological control is needed to assure long-term sustainability of poplar plantations in Serbia.

5. Conclusions

To the best of our knowledge, this is the first report of *L. populi* causing bacterial canker disease on *P.* × *euramericana* cl. "I-214" in Serbia and in SEE. It is also the first record of a bacterial disease on *P.* × *euramericana* in SEE.

Lonsdalea populi is currently the most serious pathogen affecting *P.* × *euramericana* plantations in Europe. It is also a serious threat to Serbian poplar production. Therefore, there is a need for disease management strategies that are not only economically practical, efficient, and sustainable, but also likely to be accepted by poplar growers. If the disease spreads into new areas, selection and breeding for *Lonsdalea* canker disease resistance might be such a strategy.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/10/1080/s1. Figure S1: Primers used in this study. Figure S2: Maximum-likelihood (ML) tree resulting from ML analyses of the partial 16S rRNA gene sequences (1351 bp). The bootstrap support values (ML/MP ≥ 80%) are indicated at the nodes, and the scale bar represents the number of changes. The tree was rooted to *Brenneria nigrifluens*. Figure S3: *Lonsdalea*-like colonies (marked blue) re-isolated from symptomatic tissue of *Populus* × *euramericana* clone "I-214" on tryptone soya agar. Petri dishes were incubated at 30 °C for 48 h. Table S1: Bacterial strains used for phylogenetic analyses.

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