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Bacterial etiology of leaf galls of oleander (*Nerium oleander* L.) in Poland

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Abstract: In the spring of 2022, small, regular, brownish spots of parenchymatous galls on leaves of oleander plants were observed on *Nerium oleander* L. cv. Emile Sahut. Bacterial isolates were obtained from diseased samples. The 16S rRNA sequence analysis and concatenated data set of housekeeping genes *gltA, gyrB, rpoB*, and *rpoD* showed that the obtained isolates are most closely related to *Pseudomonas savastanoi* pv. *nerii.* Pathogenicity tests on *N. oleander* L. (cv. Emile Sahut) plants confirmed the pathogenicity of the isolates. The study constitutes the first report of bacterial leaf galls caused by *P. savastanoi* pv. *nerii* in Poland.

Keywords: oleander knot; pathogenicity; phylogenetic analysis; *Pseudomonas savastanoi* pv. nerii; *gltA*, *gyrB, rpoB* and *rpoD*

Nerium oleander L. is an ornamental woody plant cultivated worldwide in botanical gardens, home gardens, spa centres and parks. Because oleander is mildly cold-tolerant (minimum winter temperatures of about 6 °C), it is more popular in warm and hot climates. Therefore, plants in cool-temperate climates in central and Eastern Europe are grown in pots and kept outdoors only during summer. Numerous diseases and pests can affect oleander plants. Of fungal diseases, Cercospora leaf spot/black blemishes (*Cercospora* sp.), Witches' broom (*Sphaeropsis tumefaciens*), anthracnose (*Colletotrichum acutatum*), and oleander dieback (*Phoma exigua* var. heteromorpha) (Ridings & Marlatt 1976; Tunali et al. 2003; Álvarez et al. 2005; Lahoz et al. 2009) are the most common. Regarding bacterial diseases, oleander leaf scorch (*Xylella fastidiosa*) and oleander knot (*Pseudomonas savastonoi* pvs. *nerii* and *savastanoi*) are the most serious noted so far (Janse et al. 1982; Caponero et al. 1995; Young 1996; Alvarez et al. 1998; Purcell et al. 1999; Bella et al. 2008; Singh et al. 2010). Although *X. fastidiosa* occurs more frequently in subtropical areas and Mediterranean countries, *Pseudomonas* spp. can readily survive and develop in countries with moderate and cool climates (Kůdela et al. 2005). So far, diseases of *Oleaceae* and *Apocynaceae* plants have not been studied in Poland. Therefore, our studies aimed to isolate and molecularly identify the causal agent of leaf spots/galls observed on oleander plants grown in Poland. Emphasising the epidemiological relevance, an unrecognised new pathogen introduced via imported plants could persist under Polish greenhouse conditions, pose a risk to other subtropical ornamental plants, and spread throughout the country.

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MATERIAL AND METHODS

Isolation of the bacteria. In the spring of 2022, diseased plants of *Nerium oleander* L. (cv. Emile Sahut), imported two months earlier from Italy, were collected at a horticultural farm near Warsaw. Small, regular brownish spots of parenchymatous galls on leaves of potted oleander plants were observed (Figure 1). Symptoms resembled a bacterial knot. From the border between healthy and diseased tissue with a gall fragment, superficially disinfected, small pieces were cut out and macerated in sterile PBS buffer (0.27% Na_2HPO_4; 0.04% NaH_2PO_4; 0.8% NaCl). After 20 min, a loopful of macerate was streaked onto King's medium B (King et al. 1954) and incubated for 48 h at 27 °C. After this time, colonies were selected and purified on the same medium. A culture of *Pseudomonas savastanoi* CFBP 1670^T was used as a reference.

Molecular characterisation and phylogenetic analysis. For molecular identification, DNA was isolated using the Genomic Mini bacterial DNA isolation kit (A&A Biotechnology, Poland) according to the manufacturer's instructions. Isolates obtained from oleander were identified by (partial) sequence analysis of 16S rRNA and the *gltA*, *gyrB, rpoB* and *rpoD* housekeeping genes (Weisburg et al. 1991; Sawada et al. 1999; Sarkar & Guttman 2004; Tayeb et al. 2005). Amplification reactions (separate for each gene) were performed in a Biometra® T3000 thermal cycler using DreamTaq Green DNA polymerase (Thermo Scientific, Lithuania) according to the conditions described in the original paper. The products were sequenced at Genomed S.A (Warsaw). The sequences obtained by automatic sequencing were assembled using the SeqMan program of the Lasergene software package (DNASTAR Inc., USA). Sequences of the 16S rRNA gene were compared with sequences deposited in the EzTaxon server database [https://www.ezbiocloud.net/ (accessed on March 2025); Yoon et al. 2017]. Sequences of *gltA*, *gyrB, rpoB* and *rpoD* housekeeping genes were compared with those deposited in NCBI GenBank [http://www.ncbi.nlm.nih.gov (accessed on March 2025)]. First, a dendrogram based on the 16S rRNA gene for oleander isolates and the closest neighbour of other species, generated by the EzTaxon server, was produced. A maximum likelihood phylogenetic tree was constructed under the Hasegawa-Kishino-Yano model (the best substitution model) using the MEGAX program (Kumar et al. 2018). Subsequently, a Maximum Likelihood phylogenetic tree based on concatenated sequences of *gltA*, *gyrB, rpoB*, and rpoD genes for all pathovars of *P. savastanoi*, using the Tamura 3-parameter evolutionary model (found to be the best substitution model), was constructed (Nei & Kumar 2000).

Pathogenicity test. The pathogenicity of the two isolates obtained was tested on *N. oleander* L. (cv. Emile Sahut) plants. The plants were inoculated by injection with a hypodermic needle with a water suspension of each isolate at a concentration of 107 cfu/mL, prepared from 48 h-old cultures grown on King's B. Leaves (main midrib) and young stems/green firm shoots were wounded, the bacterial suspension was applied, and the injection site was left until absorbed. For control, stems and leaves were injected and treated with sterilised distilled water. The plants were kept in a greenhouse at 24–26 °C and observed for symptom development for up to 3 months after inoculation.

RESULTS AND DISCUSSION

In this study, bacteria were isolated from symptoms resembling bacterial knot of oleander and identified using molecular methods. Two bacterial isolates 2464-1 and 2464-2 were obtained from *Nerium oleander* L. (cv. Emile Sahut) leaves (Figure 1).

The isolates obtained were identified by (partial) sequence analysis of 16S rRNA and the *gltA*, *gyrB, rpoB* and *rpoD* housekeeping genes. When the 16S rRNA sequences of the isolates were submitted to the EzTaxon server, they were most similar to *Pseudomonas* spp. The dendrogram based on the analysis of the 16S rRNA gene of oleander isolates and the closest neighbour of other species (Figure 2), generated by the EzTaxon server, showed that the isolates from oleander grouped with *P. savastanoi* ICMP 4352^T. When sequences of *gltA, gyrB, rpoB,* and *rpoD* housekeeping genes were submitted to the NCBI database, it was found that the oleander isolates were most similar to *P. savastanoi* pv. *savastanoi* or pv. *nerii, P. amygdali, P. syringae,* sometimes in the same percentage of identity. The maximum-likelihood tree generated from the analysis of concatenated sequences of *gltA, gyrB, rpoB*, and *rpoD* genes for oleander isolates and the closest neighbour

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Figure 1. Disease symptoms on *Nerium oleander* L. (cv. Emile Sahut)

of *P. savastanoi* pathovars showed that Polish isolates 2464-1 and 2464-2 are more closely related to *P. savastanoi* pv. *nerii* (Figure 3). Besides its presence in warm-climate countries, it has recently been reported in the Czech Republic and Ukraine (Kůdela et al. 2005; Kluchevich et al. 2018). All sequences of the 4 genes analysed for 2 isolates were deposited in the NCBI database, with the following accession numbers: *rpoD* – PV433228, PV433229, *rpoB* – PV433230, PV433231, *gyrB* – PV433232, PV433233, *gltA* – PV433234, PV433235.

The isolates caused symptoms similar to those observed on the diseased plants (Figure 2) about 70 days post-inoculation. Plants treated with water were symptomless. Similar symptoms – primary and secondary parenchymatous knots following inoculation with a bacterial knot causal agent on oleander shoots were observed by other authors (Kůdela et al. 2005; Bella et al. 2006). In contrast, isolates obtained during this study did not induce dark brown to blackened lesions as noted in the Czech Republic (Kůdela et al. 2005). To fulfil Koch's postulates, bacteria were re-isolated. Re-isolates had retained the same characteristics as the original isolates and thus fulfilled Koch's postulates.

In our case, the bacterium was imported from Italy. In Polish conditions, after the outdoor cultivation period, oleander requires wintering in bright rooms with temperatures above 0 °C. Such conditions can also favour the slow growth of bacteria and the consequent development of disease symptoms. The disease symptoms were found only on some plants, but the remaining material was most likely asymptomatically infected, as in the Czech Republic (Kůdela et al. 2005). Early detection and accurate identification of a new, previously unreported pathogen, shortly after its import from Italy, enabled the quick removal of infected plants that posed a risk to other ornamental plants.

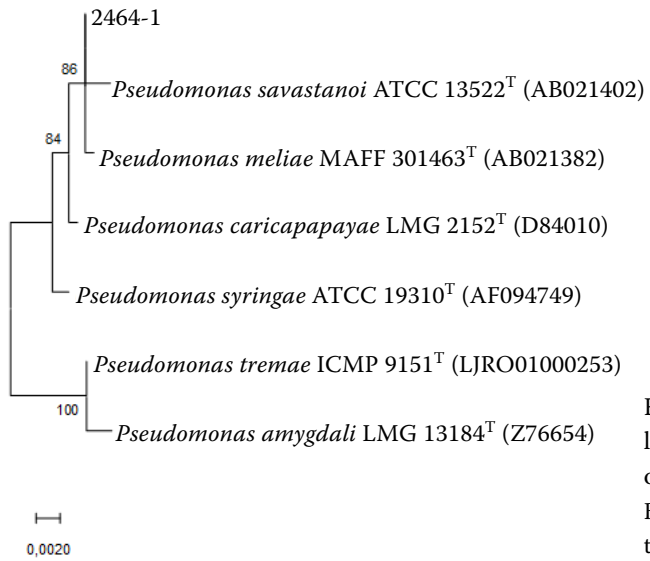

Figure 2. Maximum likelihood tree of *Pseudomonas* isolates obtained from oleander and the closest neighbor of other species, based on the of 16S RNA gene
Bootstrap values (expressed as percentages of 500 replications) are indicated at each node

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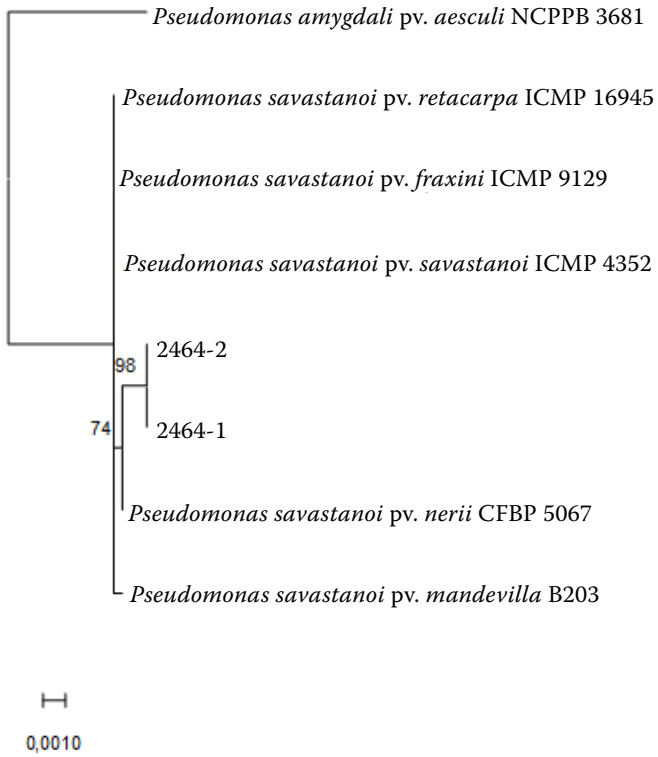

Figure 3. Maximum likelihood tree of isolates obtained from oleander and the closest neighbor of *Pseudomonas savastanoi* pathovars, based on the analysis concatenated sequences of *gltA*, *gyrB, rpoB* and *rpoD* genes
Bootstrap values (expressed as percentages of 500 replications) are indicated at each node. As an outgroup, the sequence of the same gens gene of *Pseudomonas amygdali* pv. *aesculi* NCPPB 3681 strain was used

Therefore, prevented the spread of the pathogen throughout the country during plant distribution to other localisations. The disease report also constitutes information for countries producing *N. oleander* L. for export and domestic markets on disease occurrence in nurseries. It suggests more thorough pre-sale monitoring of plants and highlights the need to develop management strategies.

CONCLUSION

As far as is known, this is the first report on the occurrence of parenchymatous galls on leaves of oleander (*N. oleander* L.) caused by *P. savastanoi* pv. *nerii* in Poland.

Our study highlights the importance of early and rapid identification of pathogens that could poten-

Figure 4. Formation of parenchymatous knots/galls following inoculation with *Pseudomonas syringae* pv. *nerii* 2464-1 on oleander shoots and leaves

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tially impact on plant health and adapt to climate conditions in our country.

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