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Editor: Anna Maria D'Onghia, CIHEAM/Mediterranean Agronomic Institute of Bari, Italy.

ORCID:

LP: 0000-0002-1850-6750

AF: 0000-0003-3399-4585

AV: 0000-0002-5942-038X

New or Unusual Disease Reports

First report of *Serratia marcescens* from oleander in Hungary

ATTILA FODOR¹, LASZLO PALKOVICS², ANITA VÉGH^{1,*}

¹ Hungarian University of Agriculture and Life Sciences, Buda Campus, Institute of Plant Protection, H-1118, Budapest Ménesi Road 44, Hungary

² Széchenyi István University, Faculty of Agriculture and Food Sciences, Department of Plant Sciences, H-9200 Mosonmagyaróvár, Vár Square 2, Hungary

*Corresponding author. E-mail: karacs.vegh.anita@uni-mate.hu

Summary. Oleander (*Nerium oleander* L.) is a popular woody ornamental plant, often used for decorating public areas, terraces and gardens. Many diseases may decrease in the ornamental value of these plantings. Between 2018 and 2020, plant pathogenic bacteria of oleander were examined, and many samples of infected plants were collected from different sites in Hungary. Two non-pigmented *Serratia marcescens* isolates were identified from oleander by classical and molecular methods. The isolates caused necrotic lesions on oleander leaves. *Serratia marcescens* is known as an opportunistic mammal or plant pathogen, but non-pathogenic strains are known to be useful biological control agents or plant growth-promoting bacteria. This is the first report of the plant pathogen *S. marcescens* from oleander, and the first identification of the bacterium in Hungary.

Keywords. Bacterial disease, PCR, morphological characterization.

INTRODUCTION

Nerium oleander (L.) is cultivated in many countries as an ornamental plant. It is native to the Mediterranean region of southern Europe and southwest Asia. In central and western Europe oleander is grown as a winter garden or patio plant. Some plant pathogenic bacteria cause serious diseases on oleander. The most widespread disease is oleander knot, caused by *Pseudomonas savastanoi* pv. *nerii* (Janse). The typical symptoms are formation of knots on stems, twigs and leaves (Bella *et al.*, 2008). *Xylella fastidiosa* (Wells *et al.*, 1987), an important plant pathogen, causes a variety of diseases with severe economic impacts for agriculture, public gardens, and the environment. Symptoms of *X. fastidiosa* on oleander are chlorotic mottling and scorching of the leaves. As the disease develops, the leaves become necrotic, and severely infected plants defoliate and die (Purcell *et al.*, 1999).

Serratia marcescens (*Enterobacteriaceae*) is a Gram-negative bacterium. *Serratia* genus includes 14 recognized species and two subspecies (Mahlen, 2011). *Serratia marcescens* was the first described member of the genus, and

was discovered by Bartolomeo Bizio in 1823 (Grimont *et al.*, 2006). *Serratia marcescens* is ubiquitous in the environment, and is commonly found in soil, water, plants, animals, humans and foods (Cooney *et al.*, 2013). It is an opportunistic mammal or plant pathogen, but non-pathogenic strains are known to be useful biological control agents or plant growth-promoting bacteria.

Serratia marcescens is also an important plant pathogenic bacterium, causing cucurbit yellow vine disease (CYVD), which was first observed in squash (*Cucurbita maxima* L.) and pumpkin (*Cucurbita pepo* L.) in the United States of America in 1988. It is now known to affect other cucurbits, including watermelon (*Citrullus lanatus* Thunb.) (Bruton *et al.*, 1998), cantaloupe melon (*Cucumis melo* var. *cantalupensis* Ser.) (Bruton *et al.*, 2003) and oil pumpkin (*Cucurbita pepo* var. *styriaca* L.) (Sedighian *et al.*, 2018). The bacterium has also been isolated from alfalfa (*Medicago saliva*) in the United States of America (Lukezic *et al.*, 1982), from onion bulbs (*Allium* sp. L.) (Coher and Dowling, 1986) in Australia, from bell pepper (*Capsicum annuum* L.) in Venezuela (Gillis *et al.*, 2014), from sunflower (*Helianthus annuus* L.) in Russia (Ignatov *et al.*, 2016), from hemp (*Cannabis sativa* L.) in the USA (Schappe *et al.*, 2019), and from ginger (*Zingiber officinale* Roscoe) in China (Huang *et al.*, 2020). However, plant-associated *S. marcescens* strains promote plant growth (George *et al.*, 2013; Matilla *et al.*, 2017; Khan *et al.*, 2017; Dutta *et al.*, 2020), and other strains are biological control agents (Someya *et al.*, 2000; Gyaneshwar *et al.*, 2001; Someya *et al.*, 2005; Queiroz and Melo, 2006; Dhar Purkayastha *et al.*, 2018), which can replace the use of chemical pesticides. A few strains can degrade herbicides (Silva *et al.*, 2007). Pigmented *S. marcescens* has been associated symbiotically with *Rhynchosporus ferrugineus* Olivier (Coleoptera: Curculionidae), as the bacterium was regularly isolated from the reproductive apparatus of weevil adults and eggs and along tissues of infested palms (Scrascia *et al.*, 2016). In addition, *S. marcescens* has been associated with disease and significant losses in Caribbean coral reefs (Sutherland *et al.*, 2011). The bacterium is also an opportunistic human pathogen causing nosocomial infections in hospitalized patients, and may spread in epidemic proportions (Hejazi and Falkiner, 1997; Matilla *et al.*, 2017; Abreo and Altier, 2019). *Serratia* species are inherently resistant to several antibiotics and can readily acquire resistance to antimicrobial agents (Mahlen, 2011).

MATERIALS AND METHODS

Eight infected plant samples were collected from Budapest's hobby gardeners between 2018 and 2020.

Symptoms of browning, necrosis and deformation were observed on the seedcases and leaves of infected oleander plants. The symptomatic plant parts were delivered to the laboratory of the Department of Plant Pathology, Hungarian University of Agriculture and Life Sciences. The samples were surface-sterilized with 75% ethanol, and small tissue pieces were cut from the margins of lesions with a sterile scalpel and then macerated in sterile distilled water (SDW). Resulting suspension was streaked onto King's B agar (King *et al.*, 1954) or Nutrient Agar (NA). The agar plates were incubated at room temperature (RT) for 48 to 72 h, and pure cultures of bacterial isolates were obtained by colony subculturing. Pure bacterial cultures, 24 to 48 h old, were used for further investigations.

Hypersensitive reaction (HR) was tested on tobacco leaves (*Nicotiana tabacum* L. cv. Xanthi) using pure bacterial suspension of 5×10^7 cells mL⁻¹, as determined with a spectrophotometer set at wavelength 560 nm. Leaves were assessed at 24 and 48 h post-inoculation. Gram feature was determined by the KOH test (Powers, 1995). Biochemical analysis was performed using the API 20E kit (BioMérieux).

For pathogenicity tests, oleander, sunflower and alfalfa plants, bell pepper fruit and onion bulbs were used. Stem internodes, between the first and the second leaves of oleander, sunflower and alfalfa, were injected using sterile syringes containing bacterial suspension of 5×10^7 cells mL⁻¹. Additionally, some leaves on each inoculated plant were lacerated with the inoculation syringe needle. Three inoculation replicates for each isolate were used. SDW was used for injection as a negative inoculation control. The inoculated plants were maintained at RT with a relative humidity greater than 90% for 1 week. Surface-disinfected bell pepper fruit and onion bulbs were toothpick-inoculated with bacterial suspensions (5×10^7 cell mL⁻¹), and were then incubated at RT with high relative humidity (>90%). Symptoms observations were carried out daily for 1 week post-inoculation. Negative controls were inoculated with SDW.

The amplification and sequencing of 16S rDNA was achieved using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') universal primer pair (Osborn *et al.*, 2000). The polymerase chain reaction (PCR) conditions were the following: initial denaturation at 94°C for 5 min, followed by 35 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 90 s, and a final extension at 72°C for 10 min. Amplification was verified on a 1% (w/v) agarose gel in 1 × TBE buffer. The PCR products were cleaned with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH), inserted into pGEM-T Easy Vector, and trans-

formed into *Escherichia coli* DH-5 α bacterium cells. The nucleotide sequence of the PCR amplified DNA fragment from the recombinant plasmids was determined and compared with sequences from the National Center for Biotechnology Information database, using the Basic Local Alignment Search Tool (BLAST) program. Homologous sequences from other known members of the *Enterobacteriaceae*, having plant or insect associations, were included in the phylogenetic analysis for comparisons.

RESULTS

Two isolates from oleander, one each from a seed-case and a leaf, were isolated from a garden in Budapest. The colonies of both isolates were non-pigmented, shiny and round with smooth surfaces on King's B agar and NA (Figure 1). The KOH tests were positive, so both isolates were Gram-negative. The isolates induced HR on tobacco leaves 24 h post-inoculation (Figure 1). Both isolates had the same biochemical attributes. They were negative for production of sodium thiosulfate, indole, acetoin, arginine hydrolase, urease, cytochrome-oxidase, inositol, rhamnose and arabinose. They were positive for β -galactosidase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, tryptophane deaminase, gelatinase, glucose, mannitol, sorbitol, saccharose, melibiose and amygdalin (Figure 2). The results of biochemical tests showed that these two isolates belonged to the *Enterobacteriaceae*.

Pathogenicity tests were positive for oleander, sunflower and alfalfa plants, bell pepper fruit and onion bulbs. Necrotic lesions were observed on oleander leaves around the points of injections, 3 weeks post-inoculation. The leaves of sunflower plants became yellow and wilted. Cross-sections of stems near the affected leaves showed brown discolorations in the phloem tissues. The plants wilted and completely died 1 week after inoculation. Alfalfa plants showed the same symptoms as sunflower plants, but symptoms of alfalfa plants developed more slowly than on sunflower plants. Control plants inoculated with SDW showed no symptoms. On bell pepper fruit, chlorotic lesions were observed around the inoculation sites within the 24 h post inoculation. Later, the lesions enlarged, turned brown and became mushy. There were sagging lesions around the inoculation points on onion bulbs, while the bulb scales also turned brown and became mushy. After 96 h, no symptoms were visible on control fruits (Figure 3). The pathogen of interest was the only microorganism re-isolated from lesions on the different inoculated plants, and was confirmed by PCR, fulfilling Koch's postulates.

The obtained nucleotide sequences were deposited into the GenBank database (accession numbers: MZ477518, MZ477519). The sequence identities were 98.9 to 99.8% to several *S. marcescens* strains, and the two isolates from oleander were shown to be 100% identical. Sequences that were used for the phylogenetic analysis were mainly derived from bacterium isolates from soil, food, insects or plants. The two isolates from oleander were least related to an isolate from corn

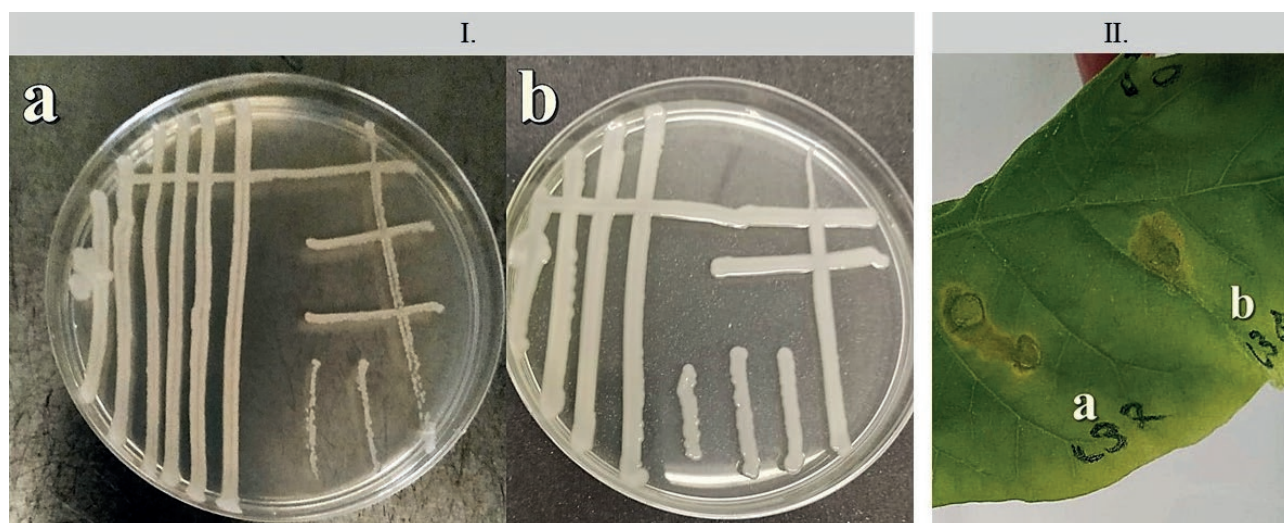


Figure 1. I. Pure cultures of bacterium colonies, after 24 to 48 h on King's B agar (a, *Serratia marcescens* isolate Bu-OleS2, and b, isolate Bu-OleS1). II. Hypersensitive reactions from infiltration of tobacco leaves 36 h post-inoculation for inoculations with, a, isolate Bu-OleS2 or b, isolate Bu-OleS1).

	ONPG	ADH	LDC	ODC	CIT	H ₂ S	URE	TDA	IND	VP	
Bu-OleS2	+	-	+	+	+	-	-	+	-	-	
Bu-OleS1	+	-	+	+	+	-	-	+	-	-	
	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX
Bu-OleS2	+	+	+	-	+	-	+	+	+	-	-
Bu-OleS1	+	+	+	-	+	-	+	+	+	-	-

Figure 2. Results from the API 20E test for two *Serratia marcescens* isolates (Bu-OleS2 and Bu-OleS1). (ONPG: β -galactosidase; ADH: arginine dihydrolase; LDC: lysine decarboxylase; ODC: ornithine decarboxylase; CIT: utilization of citrate; H₂S: hydrogen sulfide; URE: urease; TDA: tryptophan deaminase, IND: indole, VP: the Voges-Proskauer test for the detection of acetoin; GEL: gelatinase, GLU: glucose, MAN: mannose, INO: inositol, SOR: sorbitol, RHA: rhamnose, SAC: sucrose, MEL: melibiose, AMY: amygdalin, ARA: arabinose).



Figure 3. Results from pathogenicity tests for different plants, from inoculation controls (upper row), or plants inoculated with two *Serratia marcescens* isolates (Bu-OleS2 or Bu-OleS1). The first column shows oleander leaves on the day of inoculation, the second and third columns show sunflower and alfalfa plants, the fourth and fifth columns show bell pepper fruit and onion bulbs at 5 to 6 d post-inoculation.

(MK461848) and most similar to isolates from sunflowers (KT741017, KT741016, KT741020, KT741019, KT741018) (Figure 4).

DISCUSSION

The two *S. marcescens* isolates obtained from oleander in this study had non-pigmented colonies that were shiny and round with smooth surfaces on King's B agar. They were Gram-negative and induced HR on tobacco leaves 24 h post-inoculation (Bruton *et al.*, 2003; Gillis *et al.*, 2014). Both isolates had the same biochemical attributes in API 20E. These results mainly correlate to previously published descriptions, but in the study of Sedighian *et al.* (2018), acetoin production was positive

and tryptophane deaminase was negative, while Grimont and Grimont (2006) reported that mannitol fermentation was negative.

The pathogenicity tests were positive for oleander, alfalfa and sunflower plants, bell pepper fruit and onion bulbs. The two isolates caused necrotic lesions on oleander leaves, chlorotic and enlarged lesions on the bell pepper fruit and onion bulbs, which turned brown and mushy. In contrast, sunflower and alfalfa plants wilted and died. These reactions corresponded to those previously published for these hosts (Lukezic *et al.*, 1982; Cother and Dowling, 1986; Gillis *et al.*, 2014). The sequence of the 16S rDNA fragment was 98.9-99.8% identical to several *S. marcescens* strains. The present study isolates showed the nearest phylogenetic relationship to isolates of *S. marcescens* from sunflower.

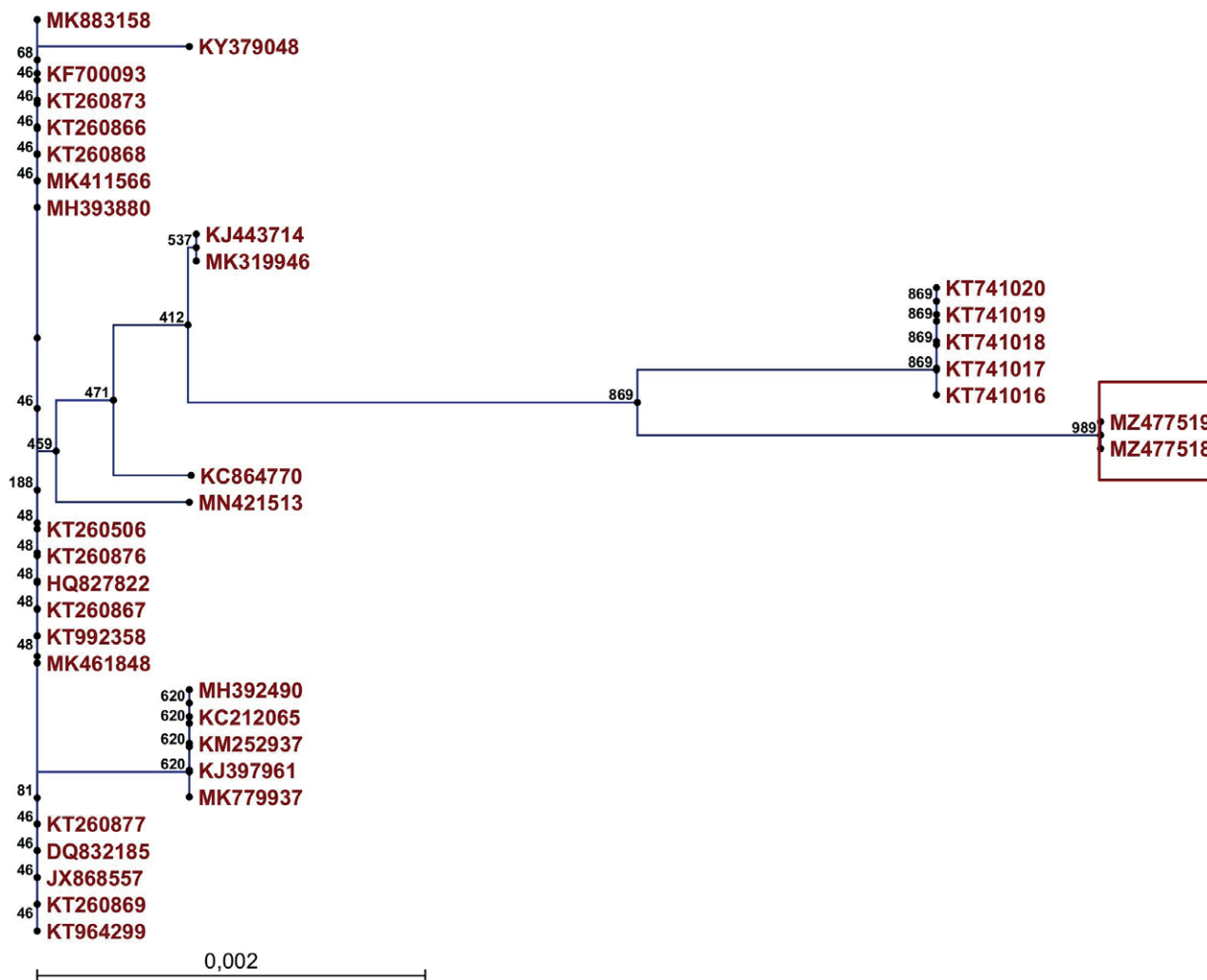


Figure 4. Phylogenetic tree of *Serratia marcescens* isolates, based on the 16S rRAS gene sequences. The tree was generated using the Neighbor-Joining method. GenBank accession numbers are shown. Hungarian *S. marcescens* isolates MZ477519 and MZ477518 are indicated with frame.

Non-pigmented strains of *S. marcescens* were previously described as the causal agent of CYVD (Bruton *et al.*, 2003; Besler and Little, 2016). However red-pigmented *S. marcescens* strains, isolated from squash in Iran (Sedighian *et al.*, 2018) or from sunflowers in Russia (Ignatov *et al.*, 2016), induced HR on tobacco leaves. The same strains were also pathogenic to zucchini (*Cucurbita pepo*). *Serratia marcescens* strains causing corn whorl rot in China produced pink or red-pigmented colonies in cultures, but HR was not reported (Wang *et al.*, 2015). In contrast, a non-pigmented *S. marcescens* strain was isolated from bell pepper in Venezuela, and it induced HR on tobacco leaves (Gillis *et al.*, 2014). The two *S. marcescens* strains from oleander were also non-pigmented and induced HR on tobacco leaves. They were also path-

ogenic to alfalfa and sunflower plants, to bell pepper fruit and to onion bulbs. Further studies are required to examine the effects of *S. marcescens* on oleander plants, since only one of the isolates assessed caused necrotic lesions, while no differences between the two isolates were observed on other hosts.

This is the first report of the *S. marcescens* as a pathogen of oleander, and the first identification of the bacterium in Hungary. The appearance of this new pathogen may cause serious problems on ornamentals, and possibly on other economically important hosts in Hungary.

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