



Periwinkle leaf spots and stem lesions caused by *Xanthomonas campestris*

Esmail Basavand · Saman Firouzianbandpey · Heshmatollah Rahimian

Accepted: 2 June 2022 / Published online: 8 August 2022
© Koninklijke Nederlandse Planteziektenkundige Vereniging 2022

Abstract A bacterial disease of periwinkle (*Catharanthus roseus*) was noted in some locations in Mazandaran province of Iran in 1995–1996 and 2016–2017. The affected periwinkle plants had brown necrotic lesions on stems and leaves often late in the growing season. Gram-negative, yellow-pigmented, rod-shaped *Xanthomonas*-like bacteria were consistently isolated from the infected samples. The isolates were confirmed as belonging to the genus *Xanthomonas* based on their phenotypic features and partial sequence of the 16S rRNA. Further characterization of the isolates by comparison of the concatenated sequences of five housekeeping genes (*gyrB*, *atpD*, *rpoD*, *dnaK*, *fyuA*), with those of *Xanthomonas* species and pathovars retrieved from GenBank, indicated their closest affiliation with *Xanthomonas campestris* sensu stricto. In a phylogram constructed by the neighbor joining method the isolates clustered with the named pathovars of *X. campestris*. Pathogenicity of the isolates was confirmed by inoculation of periwinkle seedlings, reproduction of symptoms and re-isolation of the isolates from the inoculated plants. Attempts to infect any of a set of hosts of the known pathovars of *X. campestris* sensu stricto were unsuccessful. Likewise, strains of *X. c.* pv. *campestris* and *X. c.* pv.

incana were unable to infect periwinkle plants. This is the first report on the occurrence of a periwinkle leaf spot and stem lesion disease caused by a strain of *X. campestris*.

Keywords Madagascar periwinkle · Bacterial disease · *Xanthomonas campestris* · Pathovar · Multilocus sequencing analysis

Introduction

Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don, Apocynaceae) is widely grown as an ornamental plant in home gardens and recreational sites, throughout Iran. It is also known as a medicinal plant, containing more than 130 indole derivatives and terpenes, including two anti-tumor compounds, vinblastine and vincristine, traditionally used for treatment of certain human diseases including some cancers (Yokoyama & Inomata, 1998).

A leaf spot and stem lesion disease was observed often late in the growing season on periwinkle plants in some gardens, sidewalk, and recreational areas in Babol and Sari cities in Mazandaran province, northern Iran in July to October 2016 and 2017. Symptoms consisted of circular to irregular small leaf spots, some of which expanded and coalesced to form necrotic blotches several millimeters in diameter. Severely affected leaves wilted and were abscised or remained attached to the stem. Infections extended from leaves into stems resulting in formation of deep-brown to black shiny stem lesions. The stem lesions frequently elongated, becoming several

E. Basavand (✉) · S. Firouzianbandpey
Department of Plant Pathology, Vali-e-Asr University of
Rafsanjan, Rafsanjan, Iran
e-mail: e.basavand@vru.ac.ir

H. Rahimian
Department of Plant Pathology, Sari Agricultural Sciences and
Natural Resources University, Sari, Iran

centimeters in length, and some expanding laterally leading to girdling of the shoot and wilting of the foliage distal to the canker site. In the early years of observing the disease (1995–1996), a *Xanthomonas* was consistently isolated from the stem lesions of a few affected plants in two sites and partially characterized (Rahimian & Zarei, 1998). The present study was undertaken to further characterize and identify the *Xanthomonas* isolates associated with the diseased periwinkle plants by phenotypic and pathogenicity tests and multilocus sequencing analysis (MLSA).

MLSA is increasingly used as a powerful approach for identification of bacterial strains and determination of their taxonomic position at the species and subspecies levels. MLSA of at least four or five relevant house-keeping genes has been found to be as determinative as conventional, DNA-DNA hybridization and average nucleotide identity (ANI), at the species rank in the genera *Pseudomonas* (Gomila et al., 2015; Mulet et al. 2010; Lalucat et al., 2020) and *Xanthomonas* (Bella et al., 2019, Popović et al., 2020, Vicente et al., 2017).

Materials and methods

Sampling and isolation

Surveys for the incidence of stem lesion and blight of periwinkle plants in the recreational locations in Sari and Babol were conducted from July to October 2016 and 2017. Samples of the symptomatic plants were collected and brought to the laboratory. Segments of the leaves and stems bearing the infected tissue were dipped for 15 seconds in 75% ethanol and rinsed in sterile distilled water (SDW). The segments were teased apart in drops of SDW in a Petri plate, kept under a laminar flow for 10–20 min and loopfuls of the suspension were streaked on plates of sucrose nutrient agar (nutrient agar containing 1% sucrose, SNA) and yeast dextrose calcium carbonate agar (YDC) medium (Basavand et al., 2020; Borkar, 2017). Following 4–5 days incubation at 25–28 °C, the predominant colony types were selected and restreaked on SNA to check purity. For short-term storage, isolates after 48 hr. growth were kept at 5 °C and for long-term storage colonies thereof were suspended both in SDW and in 40% glycerol and stored at 5 °C and – 80 °C, respectively. A representative isolate (PR1) has been deposited

as ABRIICC 20777 in the Agriculture Biotechnology Research Institute (ABRII).

Characterisation of bacterial isolates

The bacterial isolates (named PR1 to PR15) were subjected to standard biochemical and physiological tests including Gram-stain and oxidase reaction, production of catalase and levan, hydrolysis of esculin, gelatin, and casein, oxidative/ fermentative metabolism (OF) of glucose, production of urease and fluorescent pigment on King B medium, ability to grow at 37 °C and in 2, 3, 5, and 6% sodium chloride, among others based on the methods of Borkar (2017) and Schaad et al. (2001). Assimilation of carbon sources was assayed using the basal medium of Ayers et al. (1919) supplemented with 0.3 to 0.5% of filter-sterilized carbon compound (Borkar, 2017; Schaad et al. 2001) (Table 1). *X. campestris* pv. *campestris* ICMP 6541 (isolated in 1978 from *Brassica oleracea* L. var. *botrytis*) was used as a reference strain (Young et al., 2008).

Hypersensitivity response (HR) test

All obtained bacterial isolates (15) were grown on nutrient agar (NA) plates at 25–28 °C for two days. Then, the suspension of each isolate was prepared with a concentration of 1×10^8 colony forming unit per milliliter (CFU/ml) in SDW using a spectrophotometer (WPAS2000) ($OD_{600nm} = 0.1$). The bacterial suspensions were infiltrated (20 μ l) into the underside of the pelargonium (*Pelargonium* \times *hortorum* Baily) and tobacco leaves and plants were maintained under greenhouse condition (20–24 °C and 50% humidity). The leaves were observed for necrosis 24–72 h after inoculation (Klement et al., 1990; Rahimian, 1995). The HR test was repeated three times, and SDW was infiltrated as a negative control.

Pathogenicity tests

Periwinkle and the other test plants were raised from seeds and grown in the greenhouse (20–27 °C) in pots filled with steam-sterilized perlite-cocopeat (1:1) mix. Two-month old plants were used in pathogenicity trials. Four isolates from periwinkle, a strain of *X. campestris* pv. *incana* (ICMP 16263) and an isolate of *X. campestris* pv. *campestris* (ICMP 11053) were grown on SNA for 48 h and suspended in SDW. Suspensions were adjusted

Table 1 Phenotypic characteristics of *Xanthomonas* isolates (15) from periwinkle leaf spot and stem lesion disease

Characteristics	Reaction		Characteristics	Reaction	
	Periwinkle isolates	ICMP6541 ^a		Periwinkle isolates	ICMP6541
Gram stain	– ^b	–	Production acid from:		
Oxidative/fermentative	o	o	Arabinose	+	+
Oxidase	– ^c	–	Cellobiose	+	+
Catalase	+	+	Galactose	+	+
Arginine dihydrolase	–	–	Glucose	+	+
Phenylalanine deaminase	–	–	Mannose	+	+
Fluorescent pigment	–	–	Maltose	+	+
Mucoid growth on YDC	+	+	Mannitol	+	+
Levan formation	+	+	Melibiose	+	+
β-glucosidase	+	+	Lactose	+	+
Hydrolysis of:			Sucrose	+	+
Gelatin	+	+	Trehalose	+	+
Lecithin	+	+	Adonitol	–	–
Esculin	+	+	Arabitol	–	–
Starch	+	+	Dulcitol	–	–
Tween 80	+	+	Inositol	–	–
Urease	–	–	Raffinose	–	–
Production of H ₂ S from:			Rhamnose	–	–
cysteine	+	+	Sorbose	–	–
peptone	+	+	Salicin	–	–
na- thiosulfate	+	+	Inulin	–	–
Hypersensitive reaction on pelargonium	+	+	Utilization of:		
Growth on 3% NaCl	+	+	Acetate	+	+
Growth on 4% NaCl	–	–	Citrate	+	+
Nitrate reduction	–	–	Fumarate	+	+
Growth at 37 °C	–	–	Lactate	+	+
			Malate	+	+
			Malonate	+	+
			Benzoate	–	–
			levulinate	–	–
			D- Tartrate	–	–
			L- Tartrate	–	–
			D-Fructose	–	–
			Proline	–	–
			Serine	–	–
			L-Ornithine	–	–
			L-Arginine	–	–

Note: (a) *Xanthomonas campestris* pv. *campestris* ICMP 6541, (b) Negative reaction, (c) Positive reaction

to an optical density (OD) at 600 nm of 0.1, and then diluted 100-fold with SDW (to contain approximately 1×10^6 colony forming units per milliliter, CFU/ml). The

suspensions were injected using sterile disposable syringe fitted with hypodermic needle into the parenchyma tissue of leaves and stems. Another set of plants were

inoculated by pricking the stem at several points with sterile needle dipped into a colony of a 48-h culture. Both leaves and stems of one-to two-month-old plants of garden stock (*Matthiola incana* L. (R.Br.) cvs. Midget Mix, Lavender Lilac, Noble White, Esfahan Purple, cauliflower (*Brassica oleracea* L. var. *botrytis*), cv. White Cloud, cabbage (*B. oleracea* L. var. *capitata*) cvs. Globe Master, Green Crown, Giant, Flat Dutch, and wallflower (*Erysimum cheiri* L. Crantz) cvs. Fire King, Saffaroni, Winter Sun, were inoculated similarly; for radish (*Raphanus sativus* L.) cvs. French Breakfast, Mino Early, rapeseed (*B. napus* L.) cvs. Dwarf Essex, Hayula, turnip (*B. rapa* L.) cv. Snowball and tomato (*Lycopersicon esculentum* Mill.) cvs. Rutgers, VF12, newton and sylviana only leaves were subjected to inoculation. Collectively, three plants were inoculated with each isolate. All pathogenicity tests were repeated three times. SDW instead of inoculum was used as a control. Plants were kept at greenhouse conditions (20–24 °C and 50% humidity), fertilized and watered regularly and observed daily for symptoms development for up to 8 weeks after inoculation. Re-isolation from the inoculated plants showing symptoms was done on SNA and the identity of each isolate confirmed by colony morphology and biochemical characteristics (Basavand et al., 2021a; Borkar, 2017).

Genomic DNA preparation

Genomic DNA was extracted from isolates using the previously described method with some modifications (Basavand et al., 2021b; Ausuble et al. 1992). Cells from a 48 h-old bacterial culture on NA were suspended in 700 µL CTAB (CetyltrimethylAmmonium bromide) buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.5% CTAB), and incubated at 70 °C in a water bath for 65 min. The suspension was deproteinized with 800 µl chloroform/isoamyl alcohol (24:1 v/v) and centrifuged for 10 min at 13,000 g. A 600 µL portion of the supernatant was transferred to a sterile microtube mixed with 500 µl of cold isopropanol, kept at the –20 °C for 30 min, and centrifuged for 15 min at 13,000 g. The precipitate was washed in 1000 µl of 70% ethanol and centrifuged for 10 min at 13000 g. Precipitated DNA was air-dried at room temperature and re-suspended in 50 µL 0.01 mM Tris-0.001 mM EDTA, PH 8. Concentration and purity of DNA were determined with the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and gel electrophoresis on 1% agarose followed by staining with

0.5 µg/ml ethidium bromide and visualization under UV transilluminator. The DNA was stored at –20 °C until use.

PCR and DNA sequence analysis

The 16S rRNAs of three representative isolates (PR1, PR2 and PR7) were amplified by PCR using primer pair FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al. 1991) in a 25 µl PCR reaction containing 2.5 mM MgCl₂, 0.2 mM dNTPs, 10 pmol of each primer, 2.5 µl of 10X buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.4), 1.25 U *Taq* DNA polymerase (CinnaGen, Iran) and 1 µl of template DNA. PCR amplification was performed by an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 s, 55 °C for 1 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Multilocus sequencing analysis (MLSA) of the three isolates was performed using four housekeeping genes (*atpD*, *dnaK*, *gyrB* and *rpoD*) and the transmembrane protein gene (*fyuA*), with the primers specified by Young et al. (2008) and Simoes et al. (2007) and using the same reaction mixture as the one used for amplification of the 16S rRNA gene. PCR amplification was performed with an Applied Biosystems 9700 Thermal Cycler. PCR product (4 µL) was separated by electrophoresis on a 1% agarose gel followed by staining with 0.5 µg/ml ethidium bromide and visualization under UV transilluminator. The size of the products was evaluated using a 100 bp ladder (Gene Ruler, TMDNA Ladder Mix, Fermentas, Lithuania). PCR products were sent to Macrogen Corporation (Seoul, South Korea) and sequenced via Sanger dideoxy sequencing. The dataset was aligned using ClustalW (Thompson et al. 1994), trimmed at both ends using BioEdit (version 7.0.5.2), and compared using the Blastn program with strains available in NCBI (National Center for Biotechnology Information). The phylogenetic trees were constructed employing the neighbor joining (NJ) method and the K2P model implemented in the software package MEGA7 (Kumar et al. 2016). The robustness of the phylogenetic trees was assessed using bootstrap with 1000 replicates. The sequences were deposited in the NCBI database under the accession numbers: 16S rRNA (MW024152, MW02713, MW426199), *atpD* (MW044547, MW044548, MW429432), *gyrB* (MW044553, MW044554, MW429435), *dnaK* (MW044549, MW044550, MW429433), *fyuA*

(MW044551, MW044552, MW429434), *rpoD* (MW044555, MW044556, MW044555).

Results

Isolation from symptomatic periwinkle plants (stems and leaves) on sucrose nutrient agar (SNA) yielded bacterial colonies that were yellow, circular, convex, mucoid, and 2–3 mm in diameter after 4–5 days of incubation at 25–28 °C. Similar mucoid colonies were also formed on YDC medium. From 20 infected samples, a total of 15 single colonies were further purified by streaking on SNA (eight strains from Babol and seven strains from Sari) and following 48 h incubation at 25–28 °C were kept at 5 °C for routine use.

Phenotypic characteristics

The isolates were Gram stain- negative, aerobic, rod-shaped, motile, oxidase and arginine dihydrolase negative, catalase and β -glucosidase positive. They hydrolyzed aesculin, gelatin, starch, and Tween-80. The biochemical and nutritional characteristics of the isolates are summarized in Table 1. The isolates were phenotypically similar to *X. campestris* (Vauterin et al., 1995).

Hypersensitivity response (HR) test

All tested isolates (15) gave a necrotic reaction on pelargonium and tobacco leaves within 24 to 72 h after injection of bacterial suspensions. The negative controls inoculated with SDW developed no symptoms.

Pathogenicity

Ten to 14 days post inoculation (DPI), circular to irregular, brown chlorotic spots appeared on the leaves of the inoculated periwinkle plants (Fig. 1d). Some lesion coalesced, assuming a necrotic blotchy appearance. Typical lesions developed on the inoculated stems. The lesions were deep brown in color, oval to elliptical in shape, moderately depressed and gradually became wrinkled. Most stem lesions kept expanding both longitudinally and peripherally, and some extended into the adjoining branches. Some lesions girdled the stem causing desiccation and wilting of the branch distal to the affected site. Oozing of bacterial cells from the margin

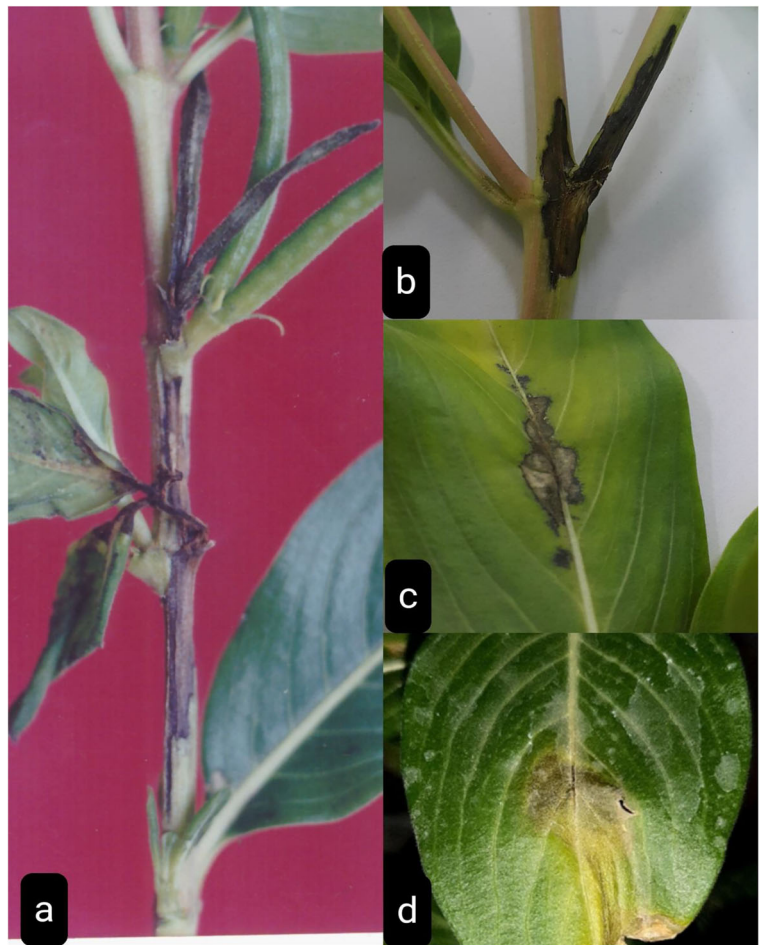
of the necrotic tissue was observed in microscopic examination of the lesions sites. The inoculated strains were re-isolated from the affected tissues, five to six weeks after inoculation and their identity confirmed by their colony morphology and reactions in a few biochemical tests. All Brassicaceae cultivars and species used became infected with *X. c. pv. campestris* 11,053 in the injection-infiltration trial and most except rapeseed cvs. and white cloud cauliflower in the prick-inoculation method. *X. c. pv. incana* ICMP 16263 produced stem lesions on all garden stock cvs. in both inoculation methods, but could not infect any other cultivar or species tested. No symptoms were observed on the control plants. The inoculated cabbage, cauliflower, garden stock, radish, wallflower, rapeseed, turnip and tomato plants did not develop any symptoms for up to two months after inoculation.

Phylogeny and identification

Partial 16S rRNA sequences of approximately 1200 bp were obtained for each strain. Blast searches in the NCBI data base (<http://www.ncbi.nlm.nih.gov>) revealed that the 16S rRNA sequences of strains isolated from periwinkle were highly similar to those of several *Xanthomonas* spp. For instance, the sequence of PR1 showed 99% identity with those of *X. campestris* pv. *campestris* strain (Xcf2-APJR), *Xanthomonas oryzae* pv. *oryzicola* (JX-2016-94), *Xanthomonas arboricola* pv. *juglandis* (APEC25) and, *Xanthomonas cucurbitae* (ATCC 23378), confirming their affiliation with *Xanthomonas*.

Partial sequences of 640, 610, 771, 551, and 561 bp for *dnaK*, *fyuA*, *gyrB*, *rpoD*, and *atpD* genes, respectively, were obtained for each strain and used in phylogenetic analyses. The total length of the alignment for the concatenated sequences of the five genes was 3134 bp. For the multilocus sequence analysis (MLSA) of these housekeeping genes NJ dendrograms of their concatenated sequences were constructed. The three periwinkle isolates formed a sub-branch in the branch occupied by the pathovars of *X. campestris* (Fig. 2). Their pairwise identity with the latter was 99%. Results of the phylogenetic analysis of the individual housekeeping genes were congruent with that of the concatenated one. The phylogram based on the sequence of *gyrB* gene is shown in Fig. 3, as a representative thereof.

Fig. 1 **a–c** Symptoms of leaf blight and stem canker caused by *X. campestris* on periwinkle, as observed in nature, **d** Symptoms of artificial inoculation after two weeks on periwinkle leaf caused by strain PR1



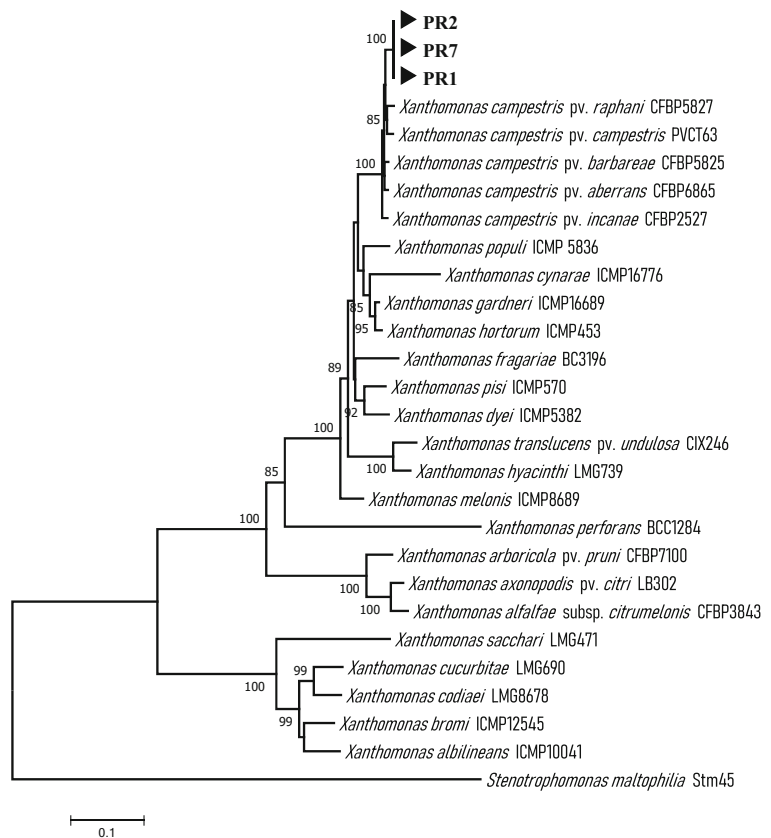
Discussion

The leaf spot and stem lesion disease of Madagascar periwinkle was observed in several locations in Mazandaran province, albeit sporadically, since 1995. A preliminary study of the disease had implicated the association of a *Xanthomonas* sp. with the disease (Rahimian & Zarei, 1998). Leaf spots of variable shapes and sizes were observed on the affected plants, but deep-brown, elongated stem lesions, which became mostly rough and wrinkled with time constituted the most characteristic symptoms of the disease (Fig. 1). *Xanthomonas* bacteria with typical yellow mucoid colonies were consistently isolated from the stem lesions and most leaf spots. Isolates possessed the general diagnostic features of *Xanthomonas* spp. in the physiological and biochemical tests as well as in the carbon source utilization

profile. Their identity as member of the genus *Xanthomonas* was confirmed in the 16S rRNA gene sequence study. Pathogenicity of the isolates on periwinkle plants was confirmed by reproduction of the typical symptoms of the disease on the inoculated plants and re-isolation of the bacterium. In pathogenicity tests several cruciferous plants and tomato which are known hosts of the validated pathovars of *X. campestris* sensu stricto (pvs. *campestris*, *incanae* and *raphani*) (Fargier & Manceau, 2007; Vicente et al., 2006) were found to be non-susceptible to the isolates pathogenic on periwinkle plants. The isolates also produced a hypersensitive reaction on pelargonium leaves which has previously found to react more swiftly than tobacco (Rahimian, 1995).

In the phylogenetic analysis based on the concatenated sequence of the housekeeping genes *gyrB*, *atpD*, *rpoD*, *dnaK*, *fyuA*, the three representative isolates

Fig. 2 Dendrogram constructed based on *rpoD*, *atpD*, *gyrB*, *dnaK*, and *fyuA* gene sequences of periwinkle isolates (PR1, PR2 and PR7) and representative strains of *Xanthomonas* spp. using neighbor-joining method. *Stenotrophomonas maltophilia* was used as the outgroup and bootstrap values (%) are marked on the branches. Bar indicates sequence dissimilarity



recovered from the affected periwinkle plants formed a highly supported sub-branch (100% bootstrap) of *X. campestris* pathovars (pvs) (Fig. 2). Based on the comprehensive study of Vauterin et al. (1995), *X. campestris* sensu stricto was confined, in its number of pathovars to only those infecting Brassicaceae species which included pvs. *aberrans*, *armoraciae*, *barbareae*, *incanae*, *raphani*, and *campestris*. More recently this number was further reduced to encompass *campestris*, *incanae* and *raphani* due to lack of adequate information in the overall characteristics and in particular on pathogenicity attributes of pvs. *aberrans*, *armoraciae* and *barbareae* (Fargier et al., 2011; Fargier & Manceau, 2007; Vicente et al., 2006). Strains isolated from blight-affected wallflower (*E. cheiri*) are considered by some workers to constitute a potential new pathovar (Vicente et al., 2001, 2006). Moreover, Koike et al., 2001 based on the inoculation experiments, suggested that the *X. campestris* strains recovered from leaf spot disease of catnip (*Nepeta cataria*, Lamiaceae) might be host specific to this plant and hence constitute

a new pathovar. The *Xanthomonas* inciting leaf spot of plantain (*Plantago lanceolata*, Plantaginaceae), formerly known *X. campestris* pv. *plantaginis*, has been found to belong to the *X. campestris* DNA homology group along with the cruciferous-infecting pathovars (Vauterin et al., 1990). The possible affiliation of this bacterium as well as some inadequately described *Xanthomonas* isolates associated with a few diseases of cruciferous and non-cruciferous weeds, including field poppy and chard, with *X. campestris* has been suggested by some workers (Parkinson et al., 2009; Ignatov et al., 2007).

Given that the isolates recovered from the affected periwinkle plants, appear to form a distinct subclade within the *X. campestris* in the MLSA phylogram, it can be inferred that they may represent a novel pathovar of this species. Their pathogenicity on a hitherto undescribed host beyond Brassicaceae by itself would support such an inference. The disease has been sporadic in occurrence in the past years (i.e., 1995–1996 and 2016–2017) never reaching an epidemic proportion or causing noticeable damage to periwinkle stands in

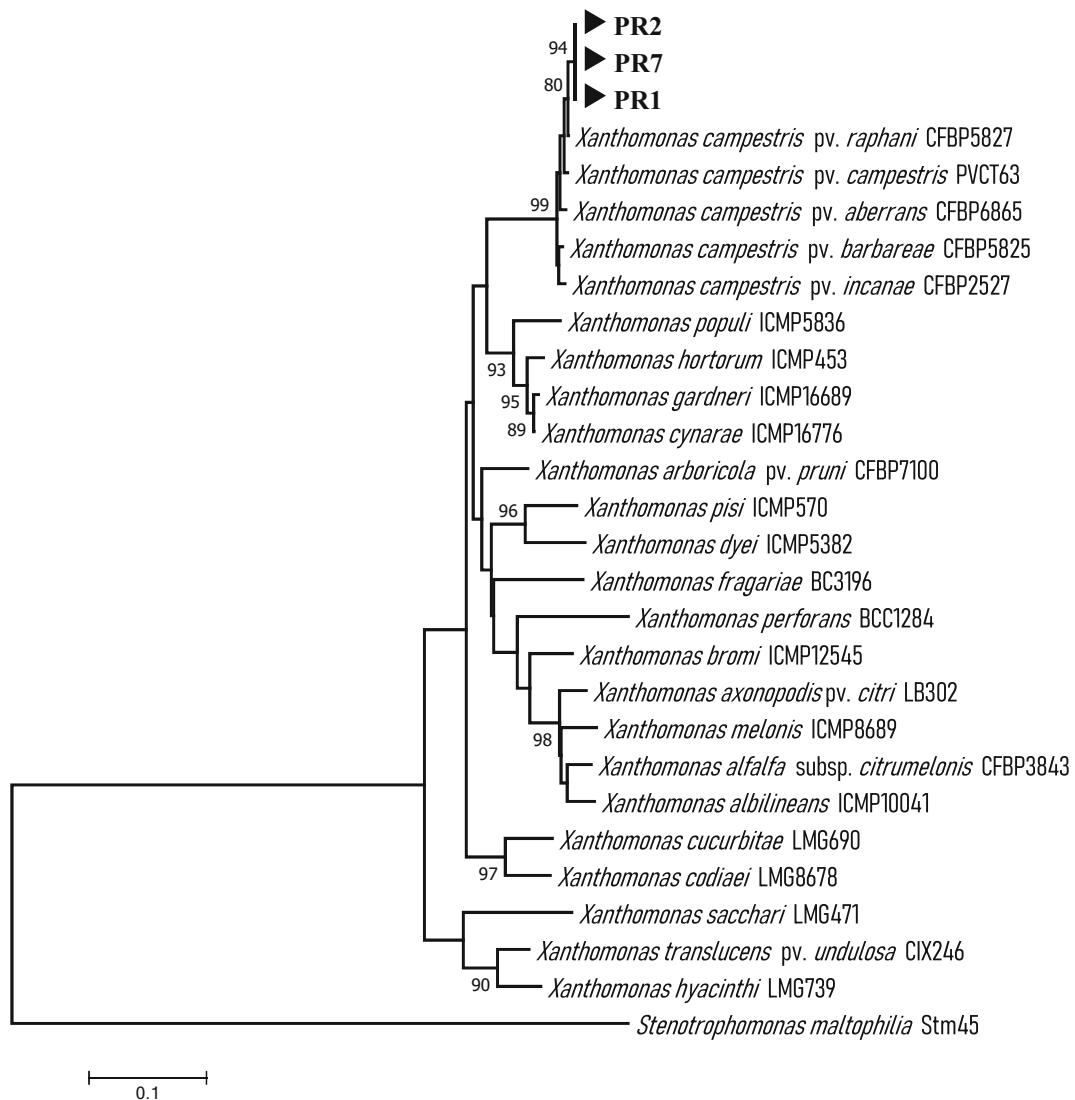


Fig. 3 Dendrogram constructed based on *gyrB* gene sequences of periwinkle isolates (PR1, PR2 and PR7) and representative strains of *Xanthomonas* spp. using neighbor-joining method.

Mazandaran overall. The disease has been more prevalent in sites irrigated by overhead sprinklers, whereas in most other locations its occurrence coincides with the onset of the rainy season in the late summer. The present report establishes the identity of the bacterium inciting the leaf spot and stem lesion of periwinkle as a strain of *X. campestris* and as a possible new pathovar of this species.

Acknowledgments The authors thank Dr. Adam J. Bogdanove and Dr. Zoë E. Dubrow for critical feedback. This work was supported by Research and Technology Office, Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

Stenotrophomonas maltophilia was used as the outgroup and bootstrap values (%) are marked on the branches. Bar indicates sequence dissimilarity

Declarations No potential conflicts of interest existed, and this study did not involve human participants or animals. All of the authors agreed to this publication.

References

- Ausuble, F., Brent, F. M., Kingstone, R. E., Moor, D. D., Smith, J. A., Seideman, J. G., & Struhl, K. (1992). *Current Protocols in Molecular Biology*. Greene, Publishing Associates, Wiley Interscience.
- Ayers, S. H., Rupp, P., & Johnson, W. T. (1919). A study of the alkali-forming bacteria found in milk. *US Department of Agriculture Bulletin*, 782, 1–39.

- Basavand, E., Khodaygan, P., Babaeizad, V., Rahimian, H., & Mirhosseini, H. A. (2020). Soft rot disease caused by *Klebsiella aerogenes* on *Austrocyliodropuntia subulate* in Iran. *Indian Phytopathology*, 73, 371–372. <https://doi.org/10.1007/s42360-020-00201-6>
- Basavand, E., Khodaygan, P., Rahimian, H., Babaeizad, V., & Mirhosseini, H. A. (2021a). *Pseudomonas syringae* pv. *syringae* as the new causal agent of cabbage leaf blight. *Journal of Phytopathology*, 169(4), 253–259. <https://doi.org/10.1111/jph.12982>
- Basavand, E., Khodaygan, P., Rahimian, H., Doonan, J. M., & Pakdin-Parizi, A. (2021b). First report of bacterial canker of fig trees caused by *Brenneria nigrifluens*. *Journal of Phytopathology*, 169(7–8), 429–437. <https://doi.org/10.1111/jph.12999>
- Bella, P., Moretti, C., Licciardello, G., Strano, C. P., Pulvirenti, A., Alaimo, S., Zaccardelli, M., Branca, F., Buonauro, R., Vicente, J. G., & Catara, V. (2019). Multilocus sequence typing analysis of Italian *Xanthomonas campestris* pv. *campestris* strains suggests the evolution of local endemic populations of the pathogen and does not correlate with race distribution. *Plant Pathology*, 68(2), 278–287. <https://doi.org/10.1111/ppa.12946>
- Borkar, S. G. (2017). *Laboratory techniques in plant bacteriology*. CRC Press.
- Fargier, E., & Manceau, C. (2007). Pathogenicity assays restrict the species *Xanthomonas campestris* into three pathovars and reveal nine races within *X. campestris* pv. *campestris*. *Plant Pathology*, 56(5), 805–818. <https://doi.org/10.1111/j.1365-3059.2007.01648.x>
- Fargier, E., Fischer-Le Saux, M., & Manceau, C. (2011). A multilocus sequence analysis of *Xanthomonas campestris* reveals a complex structure within crucifer-attacking pathovars of this species. *Systematic and Applied Microbiology*, 34(2), 156–165.
- Gomila, M., Peña, A., Mulet, M., Lalucat, J., & García-Valdés, E. (2015). Phylogenomics and systematics in *Pseudomonas*. *Frontiers in Microbiology*, 6, 214. <https://doi.org/10.3389/fmicb.2015.00214>
- Ignatov, A., Sechler, A., Schuenzel, E. L., Agarkova, I., Oliver, B., Vidaver, A. K., & Schaad, N. W. (2007). Genetic diversity in populations of *Xanthomonas campestris* pv. *campestris* in cruciferous weeds in central coastal California. *Phytopathology*, 97(7), 803–812. <https://doi.org/10.1094/PHYTO-97-7-0803>
- Klement, Z., Rudolph, K., & Sands, D. C. (1990). *Methods in Phytobacteriology* (p. 568). Akademiai.
- Koike, S. T., Azad, H. R., & Cooksey, D. A. (2001). *Xanthomonas* leaf spot of catnip: A new disease caused by a pathovar of *Xanthomonas campestris*. *Plant Disease*, 85(11), 1157–1159. <https://doi.org/10.1094/PDIS.2001.85.11.1157>
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874. <https://doi.org/10.1093/molbev/msw054>
- Lalucat, J., Mulet, M., Gomila, M., & García-Valdés, E. (2020). Genomics in bacterial taxonomy: Impact on the genus *Pseudomonas*. *Genes*, 11(2), 139. <https://doi.org/10.3390/genes11020139>
- Mulet, M., Lalucat, J., & García-Valdés, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environmental Microbiology*, 12(6), 1513–1530. <https://doi.org/10.1111/j.1462-2920.2010.02181.x>
- Parkinson, N., Cowie, C., Heeney, J., & Stead, D. (2009). Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences. *International Journal of Systematic and Evolutionary Microbiology*, 59(2), 264–274. <https://doi.org/10.1099/ijs.0.65825-0>
- Popović, T., Jelušić, A., Mitrović, P., Iličić, R., & Marković, S. (2020). Allelic profile of Serbian *Xanthomonas campestris* pv. *campestris* isolates from cabbage. *Pesticidi i fitomedicina*, 35(1), 19–26. <https://doi.org/10.2298/PIF2001019>
- Rahimian, H. (1995). Bacterial leaf spot of Zinnia in Mazandaran. *Scientific Journal of Agriculture* (Experimental Agriculture), 17(2), 1–11.
- Rahimian, H., & Zarei. (1998). Bacterial canker of *Catharanthus roseus* incited by a *Xanthomonas* sp. Proc. 13 the Iran. Plant protec. Congress, 23–26 August, Karaj, Iran, p30b.
- Schaad, N. W., Jones, J. B., & Chun, W. (2001). *Laboratory guide for the identification of plant pathogenic bacteria* (3rd ed.). American Phytopathological Society (APS press).
- Simões, T. H., Gonçalves, E. R., Rosato, Y. B., & Mehta, A. (2007). Differentiation of *Xanthomonas* species by PCR-RFLP of *rpfB* and *atpD* genes. *FEMS Microbiology Letters*, 271(1), 33–39. <https://doi.org/10.1111/j.1574-6968.2007.00691.x>
- Thompson, J. D., Higgins, G., & Ibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22(22), 4673–4680. <https://doi.org/10.1093/nar/22.22.4673>
- Vauterin, L., Swings, J., Kersters, K., Gillis, M., Mew, T. W., Schroth, M. N., Palleroni, N. J., Hildebrand, D. C., Stead, D. E., Civerolo, E. L., & Hayward, A. C. (1990). Towards an improved taxonomy of *Xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology*, 40(3), 312–316. <https://doi.org/10.1099/00207713-40-3-312>
- Vauterin, L., Hoste, B., Kersters, K., & Swings, J. (1995). Reclassification of *Xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology*, 45(3), 472–489. <https://doi.org/10.1099/00207713-45-3-472>
- Vicente, J. G., Conway, J., Roberts, S. J., & Taylor, J. D. (2001). Identification and origin of *Xanthomonas campestris* pv. *campestris* races and related pathovars. *Phytopathology*, 91(5), 492–499. <https://doi.org/10.1094/PHYTO.2001.91.5.492>
- Vicente, J. G., Everett, B., & Roberts, S. J. (2006). Identification of isolates that cause a leaf spot disease of brassicas as *Xanthomonas campestris* pv. *raphani* and pathogenic and genetic comparison with related pathovars. *Phytopathology*, 96(7), 735–745. <https://doi.org/10.1094/PHYTO-96-0735>
- Vicente, J. G., Rothwell, S., Holub, E. B., & Studholme, D. J. (2017). Pathogenic, phenotypic and molecular characterisation of *Xanthomonas nasturtii* sp. nov. and *Xanthomonas floridensis* sp. nov., new species of *Xanthomonas* associated with watercress production in Florida. *International Journal of Systematic and Evolutionary Microbiology*, 67(9), 3645–3654. <https://doi.org/10.1099/ijs.0.002189>

- Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697–703. <https://doi.org/10.1128/jb.173.2.697-703.1991>
- Yokoyama, M., & Inomata, S. (1998). *Catharanthus roseus* (periwinkle): *In vitro* culture, and high-level production of Arbutin by biotransformation. In *medicinal and aromatic plants*. Springer.
- Young, J. M., Park, D. C., Shearman, H. M., & Fargier, E. (2008). A multilocus sequence analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology*, *31*(5), 366–377. <https://doi.org/10.1016/j.syapm.2008.06.004>