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# First report of *Xanthomonas arboricola* on oleander

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**Summary.** *Nerium oleander* L is a long-lasting flowering vegetatively propagated ornamental plant of the Mediterranean region, where it is a major imported flowering pot plant. Only a few bacteria can infect it such as *Pseudomonas savastanoi* pv. *nerii*, *Agrobacterium tumefaciens* and *Xylella fastidiosa*. Between 2018 and 2022 we collected several infected plant parts in our country. In 2020 we observed atypical symptoms on the leaves and stems, which were not clearly similar to the known bacterial infection of oleander. In our work, we aimed to identify the pathogen. The isolates formed yellow-coloured bacterial colonies on King-B and on YDC agar, were Gram-negative, oxidase negative and induced hypersensitive reaction on tobacco leaves. The biochemical properties were determined by API 20E and API 50CH tests. Brown necrosis was observed on oleander leaves in a pathogenicity test. Multilocus sequence analysis was used for molecular identification of the pathogen. Three housekeeping genes (*gyrB*, *fyuA* and *rpoD*) were amplified. According to symptoms, colony morphology, biochemical features, pathogenicity and molecular methods, the pathogen was identified as *Xanthomonas arboricola.* This is the first report of the plant pathogenic *Xanthomonas* spp. on oleander.

**Keywords.** *Nerium oleander*, *Xanthomonas*, bacterial disease, Hungary.

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## INTRODUCTION

*Nerium oleander* L. is an evergreen tropical and subtropical plant of the *Apocynaceae* family. It is a vegetatively propagated ornamental plant. The plant is appreciated for its striking flower of different colours. Only a few bacteria can infect oleander during cultivation, but only *Pseudomonas savastanoi* pv. *nerii* (Janse) Young, Dye & Wilkie causes significant damage. The typical symptoms are knots or galls on stems, twigs, leaves and seedcases. The knots are few millimetres, soft and green in the early stages of infection. Then it grows to a few centimetres, turns brown (Smith, 1906) and decays as the periderm blocks the source of water and nutrients. The severity of symptoms is influenced by phytohormone production (Themsah *et*

*al.,* 2010). *Xanthomonas (*Dowson 1939*)* is a large genus of plant pathogenic Gram-negative bacteria, which are obligate aerobes and generally rod shaped with a single polar flagellum (Bradbury, 1984). Many members of this genus are yellow-pigmented because of xanthan production (Kennedy and Bradshaw, 1984). The *Xanthomonas* genus comprises 45 species (Parte *et al*., 2020) which show a high degree of host plant specificity. The range of host plants is mostly a single plant or a few plants from the same botanical family (Ryan *et al.,* 2011). However, it is difficult to define the exact range of host plants, as pathogenicity tests are only carried out on a few cultivated plants (Bull and Koike, 2015). The symptoms are ranging from water-soaked spots, wilting to cankers (Rudolph, 1993). *Xanthomonas* spp. cause serious diseases on important crops such as rice, citrus, banana, cabbage, tomato and walnut (Kennedy and Bradshaw, 1984; Ryan *et al.,* 2011). Additionally, *Xanthomonas* produces a polysaccharide called xanthan gum, which is used in industry as a thickener and emulsifier (Backer *et al.,* 1998).

## MATERIALS AND METHODS

We have been investigating oleander knot in Hungary since 2018. Only a few cases of atypical symptoms were observed during the nationwide sampling of approximately 300 samples. In 2020 two infected plant samples, one from Southern Great Plain (Kecskemét) and one from Northern Great Plain (Nyírbátor) were collected from hobby gardeners. Symptoms of brown, necrosis on the leaf, and untypical canker on the stem were observed. 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, homogenized and streaked onto King's B agar (King *et al.,* 1954). 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. Hypersensitive reaction (HR) was tested on tobacco leaves (*Nicotiana tabacum* L. Xanthi) using pure bacterial suspension of $5\times10^7$ cells $mL^{-1}$. The suspension was determined with a spectrophotometer set at $\lambda = 560$ 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 and API 50CH kit (BioMérieux). Colony morphology was observed on Yeast Extract-Dextrose-Calcium Carbonate agar (YDC). For pathogenicity test 1 year old oleander plants were used. Stem internodes, between the first and the second leaves were injected using sterile syringes containing bacterial suspension of $5\times10^7$ cells $mL^1$ and 2 leaves of each plant were also lacerated with the inoculant syringe needle. Sterilized distilled water was used for injection as a negative control. 3 plants were used for each replicate. The inoculated plants were maintained at RT with a relative humidity greater than 90% for 1 week. Symptoms were observed daily for 3-week post-inoculation. To determine host plants specificity of isolates detached leaves of apricot (*Prunus armeniaca* L.), walnut (*Juglans regia* L.), pepper (*Capsicum annum* L.*)*, kohlrabi (*Brassica oleraceae* L convar. *acephala* var. *gongylodes*), cabbage (*B. oleracea* convar. *capitata*), geranium (*Pelargonium zonale* L'Hér ex Aiton), poplars (*Populus alba* L., *P. nigra*) and willow (*Salix alba* L.) were used. The surface-sterilized leaves were infected in two ways: inoculated with bacterial colonies using sterilised toothpicks and transferred with bacterial suspension of $5\times10^7$ cells $mL^1$ using a sterilized paintbrush. Positive control leaves were infected with bacteria from Gene Bank of Institute of Plant Protection. Negative control leaves were inoculated with sterilized distilled water. The leaves were incubated at hight relative humidity. 5 leaves were used for replicates. The cabbage and the kohlrabi were bought in a supermarket and the leaves of apricot, walnut, pepper and geranium were collected in a hobby garden. Symptoms were observed daily for 2 weeks after inoculation.

Multilocus sequence analysis (MLSA) was conducted using three housekeeping genes (*fyuA*, *rpoD*, *gyrB*) and forty strains (Table 1). The polymerase chain reaction (PCR) cycling conditions for *fyuA* and *rpoD* genes were 3 min at 94°C, then 30 s at 94°C, 30 s at 54°C, 1 min at 72°C for 30 cycles, then 10 min at 72°C (Young *et al*., 2008). The PCR cycling conditions for *gyrB* were 2.5 min at 94°C, then 30 s at 94°C, 45 s at 50°C, 1 min at 68°C for 34 cycles, then 7 min at 68°C (Parkinson *et al.,* 2007). The re-isolated bacteria were confirmed by 16S rDNA PCR using 63F and 1389R primers. The PCR cycling conditions were 5 min at 94°C, then 15 s at 95°C, 30 s 55°C, 90 s 72°C for 30 cycles, then 10 min at 72°C (Osborn *et al.,* 2000). Primers for partial sequences of *fyuA*, *rpoD gyrB* and 16S rDNA are listed in Table 2. The amplification was visualised on a 1% (w/v) agarose gel in 1 × TBE buffer with ECOSafe (Biocenter). The PCR products were cleaned with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH). The nucleotide sequence of the PCR amplified DNA fragment was determined and compared with sequences from the National Center for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool (BLAST) program. Homologous sequences from other

**Table 1.** *Xanthomonas* strains referred to in this study whit collection numbers (CFBP: French Collection for Plant-associated Bacteria, ICMP: International Collection of Microorganisms, LMG: Belgian Coordinated Collections of Microorganisms, NCPPB: National Collection of Plant Pathogenic Bacteria Fera (UK)) or isolate codes (X1, X2, Xp10, KBNS163, KBNS165) and NCBI GenBank database accession numbers.

| Strain | *Xanthomonas* spp. | NCBI Accession Numbers | | |
|---|---|---|---|---|
| | | *gyrB* | *fyuA* | *rpoD* |
| X1 | *arboricola* | PQ094467 | PQ094469 | PQ094471 |
| X2 | *arboricola* | PQ094468 | PQ094470 | PQ094472 |
| CFBP 1159 | *arboricola* pv. *corylina* | EU499002 | EU498895 | EU499121 |
| CFBP 2528 | *arboricola* pv. *juglandis* | EU498951 | EU498852 | EU499070 |
| CFBP 2535 | *arboricola* pv. *pruni* | EU498853 | EU498854 | EU499072 |
| Xp10 | *arboricola* pv. *pruni* | MN520634 | MN520626 | MN520630 |
| CFBP 4924 | *axonopodis* pv. *axonopodis* | EU498952 | EU498853 | EU499071 |
| LMG 982 | *axonopodis* pv. *axonopodis* | EU498981 | EU498914 | EU499100 |
| CFBP 3836 | *axonopodis* subsp. *alfalfae* | EU499001 | EU498894 | EU499120 |
| CFBP 2524 | *axonopodis* pv. *begoniae* | EU498962 | EU498909 | EU499081 |
| ICMP 7493 | *axonopodis* pv. *citri* | EU499024 | EU498910 | EU499143 |
| ICMP 10022 | *axonopodis* pv. *citri* | EU499045 | EU498930 | EU499165 |
| CFBP 2526 | *axonopodis* pv. *glycines* | EU499003 | EU498896 | EU499122 |
| CFBP 7153 | *axonopodis* pv. *manihotis* | EU499006 | EU498898 | EU499125 |
| CFBP 6546 | *axonopodis* pv. *phaseoli* | EU499015 | EU498904 | EU499134 |
| CFBP 7663 | *axonopodis* pv. *phaseoli* | EU498968 | EU498864 | EU499087 |
| ICMP 7462 | *axonopodis* pv. *ricini* | EU499023 | EU498771 | EU499142 |
| LMG 8122 | *campestris* pv. *campestris* | EU499018 | EU498907 | EU499137 |
| CFBP 2350 | *campestris* pv. *campestris* | EU498948 | EU498849 | EU4999067 |
| CFBP 5828 | *campestris* pv. *raphani* | EU498982 | EU498877 | EU499101 |
| CFBP 7270 | *dyei* | GQ183103 | GQ183117 | GQ183090 |
| CFBP 7261 | *dyei* pv. *eucalypti* | GQ183102 | GQ183115 | GQ183088 |
| CFBP 2157 | *fragariae* | EU499000 | EU498893 | EU499119 |
| NCPPB 2949 | *fragariae* | EU499012 | EU498901 | EU499131 |
| ICMP 6646 | *fragariae* | EU499019 | EU498908 | EU499138 |
| ICMP 1661 | *hortorum* pv. *hederae* | EU498987 | EU498880 | EU499106 |
| CFBP 4925 | *hortorum* pv. *hederae* | KY984200 | KYP984167 | KYP984233 |
| KBNS163 | *hortorum* pv. *pelargoni* | KP900004 | KP899987 | KP899953 |
| KBNS165 | *hortorum* pv. *pelargoni* | KP900006 | KP899889 | KP899955 |
| ICMP 12013 | *oryzae* pv. *oryzicola* | EU499050 | EU498935 | EU499170 |
| CFBP 2286 | *oryzae* pv. *oryzicola* | EU499007 | EU498899 | EU499126 |
| ICMP 16690 | *euvesicatoria* pv. *perforans* | EU499059 | EU498944 | EU499179 |
| NCPPB 762 | *pisi* | EU498976 | EU498872 | EU499095 |
| CFBP 3123 | *populi* | EU499035 | EU498919 | EU499155 |
| NCPPB 989 | *vasicola* | EU498974 | EU498870 | EU499093 |
| CFBP 2543 | *vasicola* | EU498992 | EU498885 | EU499111 |
| ICMP 3490 | *vasicola* | EU498994 | EU498887 | EU499113 |
| NCPPB 422 | *vesicatoria* | EU498954 | EU498855 | EU499073 |
| ICMP 696 | *vesicatoria* | EU498980 | EU498876 | EU499099 |
| ICMP 115 | *vesicatoria* | EU498956 | EU498857 | EU499075 |

members of the *Xanthomonas* spp. were included in the phylogenetic analysis for comparisons. For phylogenetic analysis Mega 11.1 was used (Tamura and Nei, 1993; Tamura *et al*., 2021).

## RESULTS AND DISCUSSION

Between 2018 and 2022 we collected several infected oleander plant parts in Hungary. First of all, we brought

**Table 2.** Primers used in this study (Parkinson *et al.,* 2007; Young *et al.,* 2008).

| Sequence | Forward | Reverse |
|---|---|---|
| *fyuA* | XfyuA1F | XfyuA1R |
| | AGCTACGAYGTGCGYTACGA | GTTCACGCCRAACTGGTAG |
| *rpoD* | XrpoD1F | XrpoD1R |
| | TGGAACAGGGCTATCTGACC | CATTCYAGGTTGGTCTGRTT |
| *gyrB* | XgyrPCR2F | Xgyrrsp1 |
| | AAGCAGGGCAAGAGCGAGCTGTA | CAAGGTGCTGAAGATCTGGTC |
| 16S rDNA | 63F | 1389R |
| | CAGGCCTAACACATGCAAGTC | ACGGGCGGTGTGTACAAG |

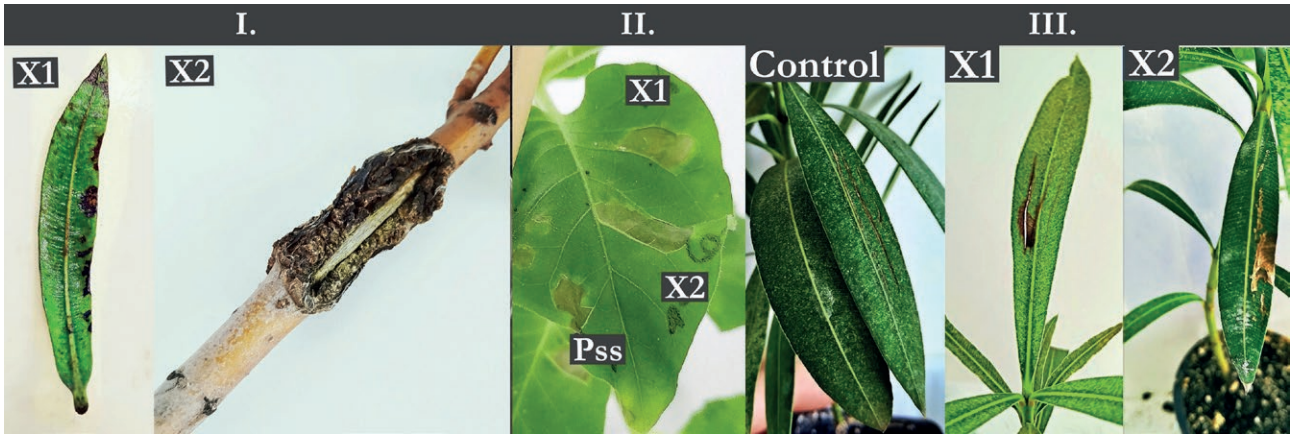


**Figure 1.** I. The symptoms of the plant parts used for isolation, II. Hypersensitive reactions from infiltration of tobacco leaves 36 h post-inoculation with negative control (*Pseudomonas syringae* pv. *syringae*-Pss) and III. Necrosis on oleander leaves around the points of injections 2 weeks post-inoculation.

into focus *Pseudomonas savastanoi* pv. *nerii*, however other pathogens (among others *Botrytis cinerea*, *Cercospora* spp.) were also investigated. In 2020 we observed two atypical symptoms, one from Southern Great Plain (Kecskemét) and one from Northern Great Plain (Nyírbátor). On the stem splitting of the canker bark were observed, which was not typical symptoms of oleander canker. In addition, there was no isolation of *Pseudomonas* spp. from the sample. On the leaf margins and main stem of the leaf scattered brown spots and watery necrosis were observed (Figure 1). It was assumed that the symptoms were caused by bacteria, which are unknown pathogens of oleander. Our previous research confirmed this hypothesis, as we isolated for the first time the pathogen *Serratia marcescens* bacteria on oleander leaf and seedcase (Fodor *et al.,* 2022). The colonies of both isolates (X1, X2) were yellow-colored, smooth-edged, slightly convex on King-B and on YDC agar. The KOH tests were positive, so both isolates were Gram-negative. The isolates induced HR on tobacco leaves 24 h post-inoculation (Kennedy and Bradshaw, 1984; Trébaol, *et al.,* 2000; Vicente and Holub, 2013) (Figure 1).

There was little variation in the biochemical properties of the isolates. The API 20NE test gave a positive reaction during sixteen reactions ($NO_3$, GLU, ESC, GEL, PNPG, GLU, ARA, MNE, MAN, NAG, MAL, GNT, CAP, ADI, MLT, CIT). Only the production of arginine dihydrolase (ADH) showed differences (X1-negative, X2-positive) (Table 3). On the API 50CH kit the isolates differed in eight tests (DXYL, ARB, SAL, CEL, MEL, TRE, GLGY, XLT). The X2 isolate showed positive results on D- Xylose, arbutin, cellobiose melibiose, trehalose, glycogen, xylitol, while the X1 isolate showed negative results. On salicin and xylitol tests of the isolate X2 showed a positive reaction, while X1 showed a negative reaction (Table 4). Vauterin and co-authors (1995) studied the biochemical properties of two *X. populi* strains. Our isolates show difference from their isolates in arabinose (ARA) utilization (positive), while in 11 reaction (NAG, CEL, GAL, MAN, TRE, SOR, TUR, XLT, GLY) our isolates were differed their isolates by half and half. The result *X. populi* of López and co-authors (2018) showed differences only in mannitol (MAN) assimilation.

**Table 3.** Result of API 20NE test ($NO_3$: nitrate reduction, TRP: tryptophan deaminase, GLU: glucose acidification, ADH: arginine dihydrolase, URE: urease, ESC: esculin ferric citrate, GEL: gelatinase, PNPG: β-galactosidase, ARA: arabinose, MNE: mannose utilization, MAN: mannitol utilization, NAG: N-acetyl glucosamine utilization, MAL: maltose utilization, GNT: gluconic acid utilization:, CAP: capric acid utilization, ADI: adipic acid utilization, MLT: malic acid utilization, CIT: citric acid utilization, PAC: phenylacetic acid utilization).

| | $NO_3$ | TRP | GLU | ADH | URE | ESC | GEL | PNPG | GLU | ARA |
|---|---|---|---|---|---|---|---|---|---|---|
| X1 | + | - | + | + | - | + | + | + | + | + |
| X2 | + | - | + | - | - | + | + | + | + | + |
| | MNE | MAN | NAG | MAL | GNT | CAP | ADI | MLT | CIT | PAC |
| X1 | + | + | + | + | + | + | + | + | + | - |
| X2 | + | + | + | + | + | + | + | + | + | - |

Two weeks after inoculation, necrosis was observed on the oleander leaves around the injection points. Control showed no symptoms. Although the isolated bacteria did not cause knots. It is assumed that the X2 isolate did not produce tumour. The pathogen of interest was the only microorganism re-isolated from lesions on the different inoculated plants, which was confirmed by colony morphology and PCR. The Koch's postulates were fulfilled only in the case of X1 isolate. The X2 isolate were also pathogen of oleander, however the symptoms experienced during isolation were not observed during pathogenicity test (Figure 1). Leaves of apricot, walnut, pepper, kohlrabi, cabbage, geranium, poplars and willow did not show symptoms. There was no difference between infected and negative control leaves. However, the positive control isolates caused necrosis around the inoculation points (Figure 2).

The phylogenetic tree was constructed using *fyuA*, *rpoD*, *gyrB* housekeeping genes, which was separated two branches. On one branch *X. oryzae*, *X. pisi*, *X. dey*, *X. vesicatoria, X. fragariae* and pathotypes of *X. axonopodis* were separated. On the other branch, *X. arboricola* and the pathotypes of *X. campestris*, *X. hortorum* and *X. populi* were found. The X1 and X2 isolate were on a separate branch with pathotype of *X. arboricola* (*X arboricola* pv. *corylina*, *X. arboricola* pv. *juglandis*, *X. arboricola* pv. *pruni*) and *X. populi*. Both isolates were similar and related to a *Xanthomonas arboricola* pv. *pruni* isolate from Montenegro (Xp10). They were on the same branch with *X. populi* (CFBP 3123). However, our isolates were different. They did not cause symptoms on *Populus* spp. and *Salix* spp. leaves, which plants are the only host of *X. populi* (De Kam, 1984). This indicates that our isolates may belong to a different pathotype, which needs to be confirmed by further studies- e.g. molecular test-

**Table 4.** Result of API 20NE test (0: Control, GLY: Glycerol, ERY: Erythritol, DARA: D-Arabinose, LARA: L-Arabinose, RIB: Ribose, DXYL: D- Xylose, LXYL: L-Xylose, ADO: Adonithol, MDX: Methyl xyloside, GAL: Galactose, GLU: D-Glucose, FRU: D-Fructose, MNE: D-mannose, SBE: Sorbose, RHA: Rhamnose, DUL: Dulcitol, INO: Inositol, MAN: Mannitol, SOR: Sorbitol, MDM: Methyl-D-mannoside, MDG: Methyl-D-glucoside, NAG: N-acetyl-glucosamine, AMY: Amygdalin, ARB: Arbutin, ESC: Esculine, SAL: Salicin, CEL: Cellobiose, MAL: Maltose, LAC: Lactose, MEL: Melibiose, SAC: Sucrose, TRE: Trehalose, INU: Inulin, MLZ: Melizitose, RAF: D-raffinose, AMD: Starch, GLGY: Glycogen, XLT: Xylitol, GEN: Gentibiose, TUR: Turanose, LYX: Lyxose, TAG: Tagatose, DFUC: D-fucose, LFUC: L-fucose, DARL: D-Arabitol, LARL: L-Arabitol, GNT: Gluconate, 2KG :2, Keto-gluconate, 5KG :5, keto-gluconate).

| | 0 | GLY | ERY | DARA | LARA | RIB | DXYL | LXYL | ADO | MDX | GAL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| X1 | - | - | - | - | - | - | - | - | - | - | + |
| X2 | - | - | - | - | - | - | + | - | - | - | + |
| | GLU | FRU | MNE | SBE | RHA | DUL | INO | MAN | SOR | MDM | MDG |
| X1 | + | + | + | - | - | - | - | - | - | - | - |
| X2 | + | + | + | - | - | - | - | - | - | - | - |
| | NAG | AMY | ARB | ESC | SAL | CEL | MAL | LAC | MEL | SAC | TRE |
| X1 | + | + | - | - | + | - | + | - | - | + | - |
| X2 | + | + | + | - | - | + | + | - | + | + | + |
| | INU | MLZ | RAF | AMD | GLGY | XLT | GEN | TUR | LYX | TAG | DFUC |
| X1 | - | - | - | + | - | + | - | - | - | - | + |
| X2 | - | - | - | + | + | - | - | - | - | - | + |
| | LFUC | DARL | LARL | GNT | 2KG | 5KG | | | | | |
| X1 | + | - | - | - | - | - | | | | | |
| X2 | + | - | - | - | - | - | | | | | |


**Figure 2.** Results from host plant specificity tests: positive controls (I.), negative control (II), inoculated with two *Xanthomonas arboricola* isolates (X2-III., X1-IV.). The first column shows cabbage, the second column kohlrabi, the third column apricot, the fourth column walnut, the fifth column pepper, the sixth column geranium, the seventh and eight column poplars, the ninth column willow leaves at 7 days post-inoculation.

ing, DNS-DNS hybridization, fatty acid analysis. To the best of our knowledge, this is the first report of the *Xanthomonas* as a pathogen of oleander. Additionally, a *Xanthomonas* spp. have been identified from olive (*Olea europaea* L), which caused brown necrosis and canker on the stream (Taylor *et al*., 2001; Young *et al*., 2010). Our isolates caused only on the oleander leaves necrosis in the pathogenicity test. They did not induce canker, although they may play a role in the evolve of on oleander knot symptoms.

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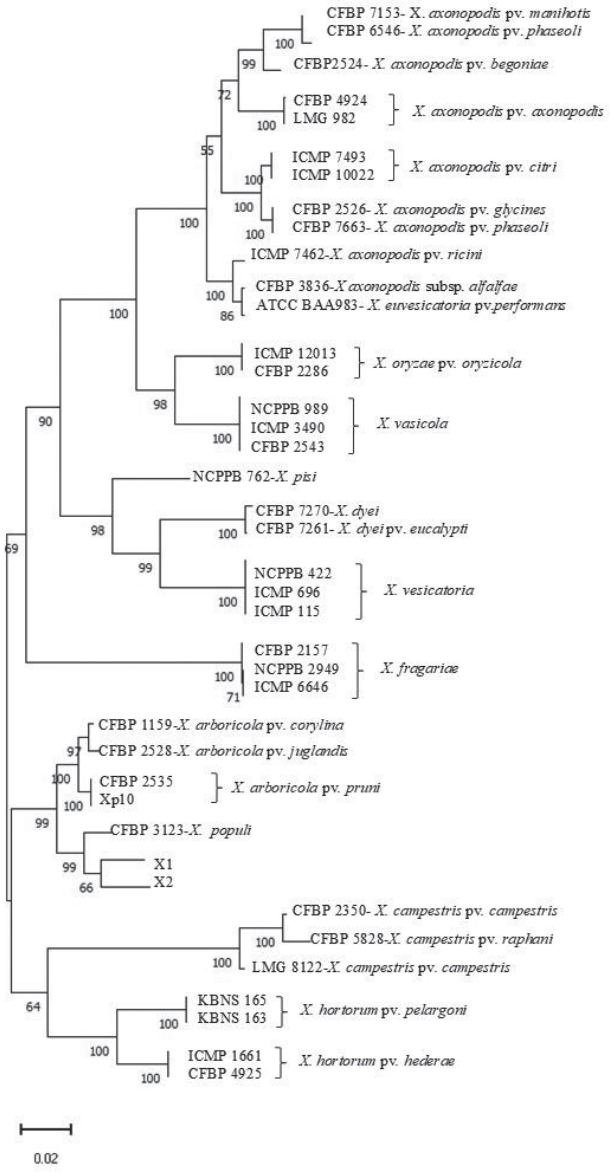


**Figure 3.** Maximum likelihood tree of concatenated *Xanthomonas* spp. nucleotide sequences for partial *fyuA*, *gyrB* and *rpoD* gene with identifier numbers. Tamura-Nei G5 model was used. The bootstrap values were 1000 samplings. The percentage of trees in which the associated taxa clustered together is shown below the branches. There were a total of 2109 positions in the final dataset (Tamura and Nei, 1993; Tamura *et al*., 2021).

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