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Research Article

Carrot slice test: A reliable method for evaluating the tumorigenicity of *Pseudomonas savastanoi* pv. *nerii*

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Summary

Pathogenicity studies on oleander plants take a long time and require significant costs to confirm the identification of isolates and to verify their pathogenicity. In this study, the carrot slice test was used as a rapid method for *Pseudomonas savastanoi* pv. *nerii*. The test is suitable for the investigation of tumorigenicity of 25 *Pseudomonas savastanoi* pv. *nerii* isolates from different parts of Hungary. On carrot slices, the first characteristic knots were observed 7 days after inoculation and were fully developed 16 days after inoculation. The one-year oleander plants used to confirm the pathogenicity of the bacteria were inoculated with a bacterial suspension to confirm the reliability of the carrot slice test. Characteristic symptoms of knots were observed on all oleander plants 46 days after inoculation. The results showed that the carrot slice method is a straightforward, rapid, and reliable method for testing and confirming tumor formation of *Pseudomonas savastanoi* pv. *nerii* without plants.

Introduction

The oleander (*Nerium oleander* L.) is a popular ornamental plant because of its abundant and long-lasting flowering. Native to the Mediterranean region, it is popular in Central and Western Europe both as a greenhouse plant and as a terrace plant. Oleander knot disease caused by *Pseudomonas savastanoi* pv. *nerii* [1,2] is the most common and dangerous disease of oleander (*Nerium oleander* L.). The typical symptoms are knots or galls on stems, twigs, leaves, and seedcases [2,3] which are incited by phytohormones [4]. The severity of the disease is mainly due to the systematic spread of the pathogen in the plant, which makes prevention and control very difficult [5]. Similar diseases occur on olive (*Olea europaea*, L) (*P. savastanoi* pv. *savastanoi* [1,2,6], on ash (*Fraxinus* spp., L) (*P. savastanoi* pv. *fraxini* [1,2,6], on Spanish broom (*Retama sphaerocarpa*, L) (*P. savastanoi* pv. *retacarpa* [7] and on other different various plants as well [2,7,8]. It is now known that the *P. savastanoi* pathotypes and *Agrobacterium radiobacter* [9] differ from most plant pathogen bacterial in that they produce plant hormones to cause knots on susceptible plants [10-12]. The youngest stages tumors caused by *A. tumefaciens* are very similar to the young knots by *P. savastanoi* pv. *nerii* [13]. For tumorigenicity tests of *Agrobacterium* spp. carrot (*Daucus carota* subsp. *sativus* L.) slices were often used [14-22]. What is more, Doksöz and Bozkurt [23] found that the carrot tumorigenicity test of *P. savastanoi* pv. *savastanoi* is a sensitive and rapid technique. Therefore, tumorigenicity tests on oleander plants are usually difficult to perform due to the large number of plants and the long time (up to 8 weeks - 10 weeks) needed to evaluate the results [5,24,25], can be replaced by carrot slices. The purpose of this study is to prove that the carrot slice test is a reliable method for evaluating the tumorigenicity of *P. savastanoi* pv. *nerii*. To verify this test, the pathogenicity test was also performed on oleander plants.

Material and methods

Bacterial strains

Infected plant samples were collected between 2018 and 2023 from hobby gardeners and public places in Hungary. 25 isolates of *P. savastanoi* pv. *nerii* (Table 1.) grown on King's

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Table 1: *P. s.* pv. *savastanoi pathovars* and *Agrobacterium* spp. isolates data.

Isolate		Country	Year	Host plant	Reference
L6	*P. s.* pv. *nerii*	Hungary	2018	*Nerium oleander*	This study
L13	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L15	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L17	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L19	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L20	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L25	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L36	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L37	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L38	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L50	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L51	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L52	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L53	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L56	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L57	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L58	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L62	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L64	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L65	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L66	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L67	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L18	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L54	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
L49	*P. s.* pv. *nerii*	Hungary	2020	*Nerium oleander*	This study
CFBP 5062	*P. s.* pv. *fraxini*	Netherlands	1978	*Fraxinus excelsior*	Iacobellis *et al.*, 1998 [33]
CFBP 5513	*P. s.* pv. *retacarpa*	Spain	1997	*Retama sphaerocarpa*	Garcia de los Rios 1999 [7]
B.01823	*P. s.* pv. *savasanoi*	Hungary	1999	*Forsythia* spp	Besenyei and Hevesi, 2003 [34]
L141	*P. s.* pv. *savastanoi*	Hungary	2023	*Olea europaea*	This study
B.01178	*Agrobacterium radiobacter*	Russia	-	-	Russian Collection of Microorganisms - VKM[1]
B.01336	*A. rubi*	USA	1950	*Euonymus* spp	isolated by Braun[2]
B.02389	*Allorhizobium vitis*	Hungary	-	*Vitis vinifera*	Szegedi, unpublished

[1]https://vkm.ru/catalog/bacte/Agrobacterium_radiobacter.htm
[2]Ophel KM, 1987. *Agrobacterium*: plasmids and biovars. University of Adelaide, Dept. of Plant Pathology, (Doctoral dissertation).

Medium B (1,5 g K_2HPO_4; 20 g pepton, 10 g glycerol, 1,5 g $MgSO_4$ X 7 H_2O, 15 g agar, 1 l H_2O, pH 7.2) for 48 h at 25 ºC were used [35]. In addition, the bacterial culture was stored at -20 ºC and -70 ºC in 20% glycerol preservative liquid for longer storage (3g Beef extract, 5 g pepton, 20 g glycerol, 1 l H_2O). The isolates were previously examined by classical methods [25,26]. An *Allorhizobium vitis* (B.02389) strain [27,28], an *Agrobacterium radiobacter* (B.01178) strain, an *A. rubi* (B.01336) strain [29,30], a *P. savastanoi* (B.01823) strain from National Collection of Agricultural and Industrial Microorganisms (NCAIM, Hungarian University of Agriculture and Life Sciences, Budapest, Hungary), a ***P. savastanoi*** pv. ***retacarpa*** (CFBP5513) strain and a ***P. savastanoi*** *pv. fraxini* (CFBP5062) strain from the French Collection for Plant-associated Bacteria (CFBP), which is a collection of the International Center for Microbial Resources (CIRM, France) and *P. savastanoi* pv. *savastanoi* strain (Table 1) were used as positive controls.

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Tumorigenicity test of carrot slices

The carrot taproots, which were bought in the supermarket, were washed under running water, next sterilised in 70% ethanol for 5 min, then washed with sterile distilled water, and finally dried on sterile paper. After sterilization, the carrot taproots were peeled, the top and bottom segments were cut off, and were sliced with a sterile scalpel to a thickness of 0.5 cm - 1 cm. Carrot slices were inoculated separately per isolate with pure bacterial cultures using a toothpick and placed in sterile Petri dishes on moistened sterile filter paper. Each carrot slice was punctured once in the middle. For each isolate (Table 1), 3 slices inoculated with bacterial culture and 2 slices inoculated with sterile distilled water were used as negative controls. Petri dishes were placed in a plant growth chamber (PHCBI MLR-352H-PE) at 80% - 85% relative humidity and 25 °C. Knots were observed until 20. days after inoculation and results were visually assessed [16,23,31].

Pathogenicity test on oleander plants

One-year-old home-grown oleander plants were used to verify the safety of the carrot slice assays. Internodes between the first and second, and third and fourth leaves of oleander plants were injected with a sterile syringe needle containing bacterial suspension at a concentration of 5×10^7 cells mL^{-1}, which was determined using a spectrophotometer set at 560 nm. Sterile distilled water was used as a negative control. Inoculated plants were kept in a greenhouse at a relative humidity above 90% for 1 week and observed 60 days after inoculation.

Identification of re-isolated pathogens

The carrot slice and parts of oleander plants were surface-sterilized with 75% ethanol and small pieces of tissues were cut with a sterile scalpel and macerated in Sterile Distilled Water (SDW). A spoonful of the resulting suspension was streaked onto King B agar [35]. The plates were incubated at room temperature (RT) for 48 hours - 72 hours and pure cultures of bacterial isolates were analyzed by PCR (Polymerase chain reaction). The amplification and sequencing of 16S rDNA was achieved using 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') universal primer pair [32]. 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 90s. The final extension was 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). The nucleotide sequence of the PCR-amplified DNA fragment was determined and compared with sequences from the National Center for Biotechnology Information (NCBI Genebank) database, using the Basic Local Alignment Search Tool (BLAST) program.

Results and discussion

All tested strains caused small, green tumors on the endodermis of the carrot slides. It was not possible to distinguish our isolates on the basis of typical knot symptoms. The first young knots on carrot slices were observed 7 days after inoculation and the typical knots developed 16 days after inoculation (Figure 1). Positive control strains showed the same symptoms as our isolates. Negative control carrot slices inoculated with sterile distilled water showed no symptoms. The pathogens were re-isolated from the knots and confirmed by PCR. The reaction of the other two pathotypes of *P. savastanoi* (*Ps* pv. *retacarpa* and *Ps* pv. *fraxini*) showed a slight delay as the symptoms appeared 9 days after inoculation, but there was no difference in symptoms. In previous studies using carrot slices to test tumorigenicity, the same results were obtained [16,23].

In the pathogenicity test on oleander plants, the first knots were formed on stems 28 days after inoculation. After 46 days the typical knots were observed on all infected plants (Figure 2). Negative control plants inoculated with sterile distilled water showed no symptoms. The pathogens were re-isolated from the knots and PCR confirmed that *Pseudomonas savastanoi* pv. *nerii* was responsible for the symptoms.

The tumorigenicity tests on carrot discs and pathogenicity tests on oleander plants resulted in similar tumors on the plant parts. The carrot slice technique is a quite simple and responsible method for testing the tumorigenicity of *P. savastanoi* pv. *nerii* without plants. It requires less space and resources, the carrot taproot is easily accessible and the time required for a pathogenicity test can be significantly reduced. This is the first report where carrot slices were used for testing the tumorigenicity of *P. savastanoi* pv. *nerri*, *P. savastanoi* pv. *fraxini* and *P. savastanoi* pv. *retacarpa*.

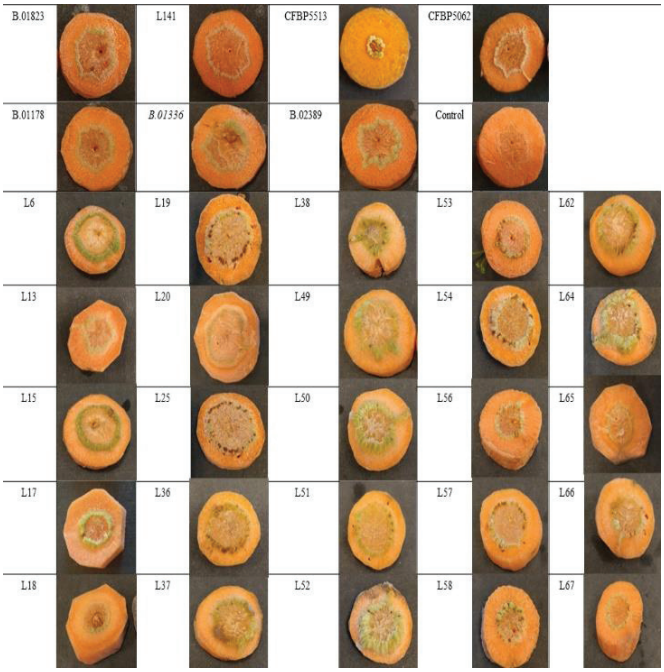

Figure 1: Typical knots on carrot slice 14–16 days after inoculation: Legend: The first row shows symptoms of other *Pseudomonas savastanoi* pathotypes, the second row shows symptoms of *Agrobacterium* spp. and absence of knot formation on sterile distilled water inoculated carrot slice, and from the third our *Pseudomonas savastanoi* pv. *nerii* isolates on carrot slices 14 days after inoculation.

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Figure 2: Tumorgenicity test on oleander stems 46 days after inoculation with *Pseudomonas savastanoi* pv. *nerii* isolates.

Conclusion

The results showed that the inoculation of carrot slices technique can be successfully applied to the tumorigenicity test of all *Pseudomonas savastanoi* pathovar. The tumorigenicity technique with the use of carrot slices may also be applicable to other tumor-forming bacteria.

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