An in vitro bioassay for evaluating the virulence of *Pseudomonas savastanoi* pv *savastanoi* isolates on olive

Mazen Salman¹ · Ali McClean² · Daniel Kluepfel²

Received: 31 May 2021 / Accepted: 4 January 2022 / Published online: 7 January 2022
© The Author(s) under exclusive licence to Australasian Plant Pathology Society Inc. 2022

Abstract

Pseudomonas savastanoi pv. *savastanoi* causes olive knot disease that afflicts olive trees globally. A sensitive and reproducible in vitro pathogenicity bioassay using detached leaf segments was developed after isolation of Psav on KB medium amended with vancomycin, cephalexin and cycloheximide. Symptoms were clear under 20X magnification after 10 days. This bioassay will facilitate rapid screening of olive germplasm collection for resistance to Psav.

Keywords *Olea europaea* · Olive knot · Resistance · Susceptibility · Virulence

Pseudomonas savastanoi pv. *savastanoi* (Psav) is the causal agent of olive knot on *Olea europaea* L. in many parts of the world (Salman et al. 2020; Hall et al. 2004; Gardan et al. 1992). Infection by Psav is known to reduce yield, quality and tree longevity (Krid et al. 2009). Symptoms of the disease are characterized by knots or galls on different parts of the tree, mainly young branches, twigs and stems but occasionally on fruits and leaves (Moretti et al. 2008). Knot development is mainly due to the production of indole-3-acetic acid (IAA) and cytokinins by the bacterium (Surico et al. 1985; Nester & Kosuge 1981; Comai & Kosuge 1980).

Reliable diagnosis and identification of Psav depends on the isolation, characterization and identification of the bacteria (Young 2004). Psav identification requires time-consuming pathogenicity tests and biochemical or molecular biology methods (Penyalver et al. 2000). Pathogenicity tests are usually achieved by infecting young stems of susceptible olive trees with a bacterial suspension followed by incubation of the plants in the green house until development of symptoms (Young 2004). Symptom development to the point of developing knots large enough to be detected by the

human eye can take many weeks to months making these bioassays less than ideal.

Due to increasing interest in the development of rapid, practical and effective methods for studying olive knot disease, we report here an enhanced method for the efficient isolation of Psav from olive knot (OK). We have also developed a novel and rapid in vitro pathogenicity test using detached olive leaf segments.

Pseudomonas savastanoi pv *savastanoi* was isolated from knots (about 1 cm diameter) collected from different olive cultivars grown in the USDA National Clonal Germplasm Repository (NCGR) olive collection located at the Wolf-skill Experimental Orchard (WEO) in Winters, CA, USA (Salman et al. 2020). The knots were surface disinfected between two layers of a paper towel moistened with 70% ethanol for 30 s. The outer surfaces of the knots were then removed with a sterile scalpel. The internal tissues were cut into small pieces (0.5–1.0 mm) and placed individually in 100 µl of sterile distilled water for 15–20 min. A loopful of each bacterial suspension was then streaked on plates containing King's medium B (King et al. 1954), supplemented with cephalexin 50 µg ml⁻¹, vancomycin 150 µg ml⁻¹ and cycloheximide 50 µg ml⁻¹ (KBvc media). The plates were incubated at 25 °C for 2 days (Krid et al. 2009; Marchi et al. 2009). Bacterial isolates were identified as Psav by amplification of the *iaaL* gene, using Psav specific IAALF and IAALR primers (Penyalver et al. 2000).

A quick and non-destructive pathogenicity test was performed using different olive cultivars (Table 1) as mentioned by Bella et al. (2006). Young symptomless leaves (one year

✉ Mazen Salman
m.salman@ptuk.edu.ps

¹ College of Agricultural Science and Technology, Palestine Technical University-Kadoorie, Tulkarm, State of Palestine

² USDA-ARS, Crops Pathology and Genetics Research Unit, University of California, Davis, CA 95616, USA

Fig. 1 Detached leaf assay showing the method of artificially wounding of the midvein of olive leaf segments before inoculation with 5 μl of a 10^8 cfu ml^{-1} Psav cell suspension

old) were cut laterally into 1.5 cm segments using a sterile scalpel and each segment was wounded on the midvein with a sterile needle (Fig. 1). Five μl of suspension containing Psav isolate 12F cells (10^8 cfu ml^{-1}) from an overnight bacterial culture was transferred directly to the wound. Control leaf segments were inoculated with 5 μl sterile distilled water. Inoculated pieces (3 segments) were then placed on a cover of Petri dishes containing 0.5% water agar supplemented with 50 $\mu\text{g ml}^{-1}$ cycloheximide to prevent fungal contamination. The plates were sealed with parafilm and incubated in a 16/8 h day/night photoperiod at 28 °C. Olive knot was assessed 10 days post infection under dissecting microscope at 40 X magnification. The experiment was done in triplicate and repeated three times.

Statistical analysis was performed using XLStat (Adinsoft). Significant differences were computed after Tukey HSD test using ANOVA at $p < 0.05$. Means with different letters are significantly different.

Visual examination of the selective medium KB_{cvc} after incubation for 48 h showed no contamination by other microorganisms. All colonies were identical and resembled the morphology of *Pseudomonas* sp. using our improved Psav selective medium. Seventy-eight Psav isolates were obtained from knots collected from 78 olive trees representing 46 different olive genotypes. No differences in colony size, color and morphology were observed among the isolates. The identity of the putative Psav colonies was confirmed by PCR using IAAL primers which generated the expected 454 bp PCR product.

Small knots (<0.5 mm diameter) were observed on detached olive leaf segments 10 days post- inoculation. Larger 1–5 mm in diameter knots (Fig. 2) were observed 30 days after inoculation. The pathogenic Psav strain 12F was tested against different olive genotypes which showed a wide range of knot sizes (0.5–9.31 mm in diameter) on leaves from different olive genotypes (Table 1).

Isolation of pure Psav was enhanced by the use of KB_{cvc} . This semi-selective medium did not inhibit the growth of all other microorganisms, but surface sterilization of the knots prior to Psav isolation dramatically lowered contamination levels. Compared to reports describing new media for isolation of Psav such as OKA (Azad & Cooksey 1995) and PV-1 media (Surico and Lavermicocca 1989), our medium is

Fig. 2 Olive knots developed 10 days post inoculation with 5 μl of a 10^8 cfu ml^{-1} Psav isolate 12F cell suspension on detached olive leaf segments of different olive genotypes. **A**; Barouni, **B**; No. 12 Sevilano, **C**; Ascolana Tenera, **D**; Redding Picholine, **E**; Barnea, **F**; Arbussana, **G**; Manzanillo; **H**; Control Manzanillo leaf segment inoculated with sterile distilled water

Table 1 Olive knot sizes caused by Psav 12F on leaf segments of different olive genotypes. Data followed by different letters are significantly different after Tukey HSD test using ANOVA at $p < 0.05$

Genotype	Accession #	Means of knot sizes (mm) $\pm$ SE
Barouni	DOLE111.0000E	6.597 a $\pm$ 0.910
No. 12 Sevillano	DOLE164.0000D	3.820 ab $\pm$ 0.258
Ascolana Tenera	DOLE28.0000D	2.650 bc $\pm$ 0.338
Redding Picholine	DOLE71.0000D	2.396 c $\pm$ 0.298
Barnea	DOLE65.0000D	1.848 c $\pm$ 0.465
Arbussana	DOLE186.0000D	1.585 c $\pm$ 0.252
Bouquetier	DOLE106.0000D	0.000 d
Picholine Marocaine	DOLE25.0000D	0.000 d
Souri	DOLE23.0000E	0.000 d

simple, easy to prepare, inexpensive and effective for a wide range of genetically diverse *P. savastanoi* strains. In addition, the medium enhanced the growth of Psav so that bacterial growth was detected in less than 24 h vs 2–7 days. These results indicate the utility of the improved selective KB_{evc} medium for facile isolation of Psav strains from olive. The in vitro inoculation test provides a simple and rapid method to evaluate Psav pathogenicity (Bella et al. 2006). The new technique will facilitate the study of interactions between Psav and olive trees. To our knowledge, this is the first report of an in vitro virulence test for olive knot disease using detached olive leaves. Pathogenic Psav were able to infect leaf segments and produce visible symptoms 10 days post inoculation, whereas other in vivo systems require at least 30 days to produce visible olive knot symptoms. Most studies that test the interaction of Psav with olive trees requires longer times to assess knot development (Marchi et al. 2009; Penyalver et al. 2006). In addition, we found that development of knots on olive seedlings grown in greenhouse was visible after 3 months of inoculation (data not shown). The test will enable faster screening of large numbers of Psav strains for virulence on olive and also hasten screening of diverse olive cultivars for resistance to olive knot disease.

The olive knot disease is difficult to control by means of bactericides. The use of resistant cultivars might be an alternative approach. However, little information is available about olive resistance to the disease. Screening for resistance is a crucial step toward effective management strategies. Our in vitro pathogenicity test could allow rapid screening of large numbers of olive cultivars with investigation the interaction of the bacteria with the host plant as well as mutant or wild type bacterial strains.

Acknowledgements The financial support from Arab Fund Fellowship Program to the first author is highly appreciated. The work was also partially supported by USDA CRIS project 2032-22000-015-00D

Declarations

Conflict of interest The authors declare no potential conflict of interest.

References

- Azad HR, Cooksey D (1995) Semiselective medium for detecting epiphytic and systemic populatoins of *Pseudomonas savastanoi* form Oleander. *Phytopahtology* 85:740–745
- Bella P, Catara V, Guarino C, Cirvilleri G (2006) Evaluation of oleander accessions for resistance to *Pseudomonas savastanoi* pv. *nerii*. *J Plant Pathol* 88:273–278. <https://doi.org/10.4454/jpp.v88i3.872>
- Comai L, Kosuge T (1980) Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. *J Bacteriol* 143:950–957. <https://doi.org/10.1128/JB.143.2.950-957.1980>
- Gardan L, Bollet C, Ghorrah MA et al (1992) DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. *Int J Syst Bacteriol* 42:606–612. <https://doi.org/10.1099/00207713-42-4-606>
- Hall BH, Cother EJ, Whattam M et al (2004) First report of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi* on olives (*Olea europaea*) in Australia. *Australas Plant Pathol* 33:433–436. <https://doi.org/10.1071/AP04031>
- King EO, Ward MK, Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* 44:301–307
- Krid S, Rhouma A, Quesada JM et al (2009) Delineation of *Pseudomonas savastanoi* pv. *savastanoi* strains isolated in Tunisia by random-amplified polymorphic DNA analysis. *J Appl Microbiol* 106:886–894. <https://doi.org/10.1111/j.1365-2672.2008.04058.x>
- Marchi G, Mori B, Pollacci P et al (2009) Systemic spread of *Pseudomonas savastanoi* pv. *savastanoi* in olive explants. *Plant Pathol* 58:152–158. <https://doi.org/10.1111/j.1365-3059.2008.01935.x>
- Moretti C, Ferrante P, Hosni T et al (2008) Characterization of *Pseudomonas savastanoi* pv. *savastanoi* strains collected from olive trees in different countries. *Pseudomonas syringae* pathovars relat Pathog – Identification, Epidemiol Genomics 321–329. https://doi.org/10.1007/978-1-4020-6901-7_33
- Nester EW, Kosuge T (1981) Plasmids specifying plant hyperplasias. *Annu Rev Microbiol* 35:531–565. <https://doi.org/10.1146/annurev.mi.35.100181.002531>
- Penyalver R, García A, Ferrer A et al (2000) Detection of *Pseudomonas savastanoi* pv. *savastanoi* in olive plants by enrichment and PCR. *Appl Environ Microbiol* 66:2673–2677. <https://doi.org/10.1128/AEM.66.6.2673-2677.2000>
- Penyalver R, García A, Ferrer A et al (2006) Factors affecting *Pseudomonas savastanoi* pv. *savastanoi* plant inoculations and their use for evaluation of olive cultivar susceptibility. *Phytopathology* 96:313–319. <https://doi.org/10.1094/PHYTO-96-0313>
- Salman M, Greenhut R, Preece J et al (2020) Field evaluation of olive (*Olea europaea*) genotypes for resistance to *Pseudomonas savastanoi* pv. *savastanoi*. *J Plant Pathol* 102:663–670. <https://doi.org/10.1007/s42161-020-00549-8>
- Surico G, Iacobellis NS, Sisto A (1985) Studies on the role of indole-3-acetic acid and cytokinins in the formation of knots on olive and oleander plants by *Pseudomonas syringae* pv. *savastanoi*.

- Physiol Plant Pathol 26:309–320. [https://doi.org/10.1016/0048-4059\(85\)90006-2](https://doi.org/10.1016/0048-4059(85)90006-2)
- Surico G, Lavermicocca P (1989) A semiselective medium for detection of *Pseudomonas syringae* pv. *savastanoi*. Phytopathology 79:185–190
- Young JM (2004) Olive knot and its pathogens. Australas Plant Pathol 33:33–39. <https://doi.org/10.1071/AP03074>