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EVALUATION OF OLEANDER ACCESSIONS FOR RESISTANCE TO *PSEUDOMONAS SAVASTANOI* pv. *NERII*

P. Bella[1], V. Catara[2], C. Guarino[1] and G. Cirvilleri[2]

[1] Parco Scientifico e Tecnologico della Sicilia, Z.I., Blocco Palma I, Catania, Italy
[2] Dipartimento di Scienze e Tecnologie Fitosanitarie - sez. Patologia vegetale, Via S. Sofia, 100, Catania, Italy

SUMMARY

Nine oleander accessions were evaluated for resistance to *Pseudomonas savastanoi* pv. *nerii*, causal agent of the oleander knot disease. None of the accessions was resistant when tested with three bacterial strains of different virulence, but they varied significantly in the severity of symptoms induced by these strains. The most susceptible accessions "Dark Salmon" (dark salmon flower) showed deformation of stems, leaves and seed pods and secondary knots on aerial parts, whereas the least susceptible one "White" (white flower), inoculated with the least virulent strain, showed neither localized knots at the inoculation point nor secondary symptoms. In this study an *in vitro* test, based on prick inoculation of leaf segments, was optimised for use in pathogenicity tests or as an *in vitro* resistance screening test.

Key words: Nerium oleander, Oleander knot disease, screening for resistance, detached leaf assay.

INTRODUCTION

Nerium oleander is cultivated worldwide as an ornamental plant. It is native only in the Mediterranean region of southern Europe and southwest Asia, but it naturalizes very easily and in many areas the plant is subspontaneous. *N. oleander* is widely cultivated, particularly in warm temperate and subtropical regions where it grows outdoors in parks, gardens and along roadsides. In central and western Europe it is grown as a conservatory or patio plant.

The Tentative Checklist of Oleander Cultivars (Pagen, 1988) reports 401 distinct cultivars, 145 of them currently available in trade. The nomenclature is not uniform and no international registration authority exists: the oleander cultivars vary in form, colour and scent of flowers and in habit (Pagen, 1988). In Sicily there is a wide variety of oleander cultivars often differentiated on the basis of form and colour of flowers (Romano *et al.*, 2003).

The oleander knot disease caused by *Pseudomonas savastanoi* pv. *nerii* is one of the most widespread oleander diseases. As in the olive knot disease, the typical symptom is the formation of knots on stems, twigs and leaves incited by phytohormones produced by the bacterium (Surico and Iacobellis, 1992). The bacterium also deforms the inflorescences and seed pods, and/or reduces blooming, and causes death of pistils (Smith, 1928). The severity of the disease is mainly due to invasion of the laticifers by the pathogen, causing extensive development of secondary symptoms all over the plant (Wilson and Magie, 1964).

Systemic invasion of the plant severely compromises its aesthetic value, making the knot disease a severe threat. Moreover, once established, the bacterium is impossible to eradicate and its presence as an endophyte can lead to the dissemination of the inoculum amongst asymptomatic cuttings and plantlets.

The disease is difficult to prevent and control because of lack of effective bactericides, so selection or development of resistant cultivars is likely to be the best approach to control. Presently nothing is known about oleander cultivars with differential resistance to *P. savastanoi* pv. *nerii.* A survey conducted in the South of Italy showed that most plants are affected and that in some cultivars the entire blooming or fruiting was compromised (Bella *et al.*, 2001). The occurrence of the disease in rooted cuttings in a production nursery in Sicily made a further investigation of the oleander/*P. savastanoi* pv. *nerii* system necessary.

In this study we evaluated the resistance of some commercial oleander accessions to *P. savastanoi* pv. *nerii* strains of different virulence. Furthermore we optimised an *in vitro* pathogenicity test, which allows rapid screening of strains and/or germplasm.

MATERIALS AND METHODS

Plant material and bacterial isolates. Nine oleander accessions were obtained from one of the most impor-

Corresponding author: P. Bella
Fax: +39.095.292730
E-mail: patrizia.bella@tiscali.it

tant oleander nurseries of Eastern Sicily. The plants were one-year-old rooted cuttings, and were grown in pots (18x14 cm) (two rooted cuttings/pot) and maintained in a greenhouse during the trial.

Ten *P. savastanoi* pv. *nerii* strains were used. Nine from the collection of Dipartimento di Scienze e Tecnologie Fitosanitarie, University of Catania, were obtained during a survey in Sicily and they were shown to have different virulence. Three groups of strains were defined on the basis of symptom severity on oleander: group I induced severe cankerous necrotic lesions on stems, secondary knots on leaves and bottle shaped seed pods; group II caused local knots at the inoculation site; group III did not induce symptoms at the inoculation point but water-soaked areas on terminal leaves were observed (Bella *et al.*, 2001). The tenth strain was the reference strain ITM519, also used in several previous studies on *P. savastanoi* (Mugnai *et al.,* 1993; Surico, 1993; Caponero *et al.*, 1995). Bacteria were grown at 27 ± 2°C in King's B medium (KB) for 48h.

Screening of oleander accessions *in vivo.* Three *P. savastanoi* pv. *nerii* strains, from the most to the least virulent strain, IPVCT89.1.1 (group I), IPVCT83 (group II) and IPVCT99.2.3 (group III) were inoculated on ten plants per accession per strain. Young stems of one-year-old oleander plants were prick-inoculated in three internodes per plant, using a needle contaminated with a single bacterial colony taken from 48 hr-old cultures on KB. Control plants were pricked with a sterile needle.

Symptoms were recorded at 10, 20, 30, 60 and 120 days after inoculation. Host-pathogen interaction was evaluated on the basis of primary symptoms caused by prick-inoculation on the stem using a 0-4 scale of susceptibility: 0 = no symptoms; 1 = localized knots at the inoculation point; 2 = deformation of stem; 3 = deformation of stem and apex death. The presence of secondary knots on leaves, flowers and pods was also recorded and one point per affected organ was added to the class value. The scores of individual plants were used to calculate the disease index, DI: Σ (Class x no. of plants in each class) /no. of inoculated plants. ANOVA two-way analysis was performed on the mean disease index at the end of the experiment, i.e. four months after inoculation and the Student-Newman-Keuls test was calculated (P= 0.05). The DI values at each score registration (20, 30, 60 and 120 days after inoculation) were used to calculate the area under the disease progress curve (AUDPC) according to the equation of Campbell and Madden (1990): $AUDPC= \Sigma_i^{n-1}(y+y_{i+1})/2x(t_{i+1}-t)$, where n is the number of evaluations, y the DI, and t the number of days after *P. savastanoi* pv. *nerii* inoculation was performed.

Infection of plants was confirmed by isolation of the bacterium from plant parts showing secondary symptoms far from inoculation points, i.e. secondary knots and deformation of stem, apex and pods. Pieces of affected tissues were surface-disinfected in household bleach (diluted 1:10 V/V) for 1 min, then ground in sterile distilled water. The resulting bacterial suspension was streaked on King's B medium and plates were incubated for 48 h at 27°C. Resulting colonies were observed for typical morphology and fluorescence under UV light. When the morphology was doubtful a PCR targeted to the *iaaL* gene, as described by Penalver *et al.* (2000), was performed using a colony picked from the plate with a pipette tip.

Development of an *in vitro* pathogenicity test. Different oleander organs were evaluated to develop a quick and non-destructive pathogenicity test for the disease. Ten *P. savastanoi* pv. *nerii* strains were used. For the trial the accession "Pink" (pink single flower) was used. Young stems portions and leaves were washed under tap water for 5 min. and surface-sterilised in 70% ethanol for 60 sec., followed by one rinse with sterile distilled water. Stem segments cut into either 0.5 or 1.5 cm pieces (9 and 6 stem segments per strain respectively) were used. When using the leaves, these were cut into 1.5 cm pieces with a sterile scalpel, obtaining 5 segments from each leaf. Stems were inoculated by transferring bacterial cells directly to the exposed cut surface whereas leaves were inoculated in the midvein by needle prick. Inoculated pieces were incubated on 0.5% water agar at a 16/8 h day/night photoperiod at 28°C. Symptom development was monitored weekly. Positive inoculation sites were counted at the end of the assay. For the leaf assay a disease index (DI) was calculated per strain on the basis of the number of positive inoculation sites and of the knot size using an arbitrary scale: Class 0, no knots; 1, ≤ 1 mm; 2, 1-3 mm; 3, > 3 mm. Results were expressed as disease index, DI: Σ (Class x no. segments in each class) /no. of segments inoculated. The DI values were analysed by ANOVA and the average DI values compared by Student-Newman-Keuls test (P = 0.05).

The detached leaf assay was also used to evaluate the accession "Pink" together with the most and the least susceptible accession of the *in vivo* trial ("Dark Salmon" and "White"). *P. savastanoi* pv. *nerii* strains, IPVCT87.1.1 (group I) and IPVCT93.1.4 (group III), were inoculated on 12 pieces of detached leaves for each accession. Leaves were processed and inoculated as previously described. Results, expressed as number of positive inoculations and knots size, were scored 7, 15 and 30 days after inoculation, and the DI was calculated.

RESULTS

Response of oleander accessions to *in vivo* inoculation of *P. savastanoi* pv. *nerii*. The development of symptoms caused by inoculation is shown in Fig. 1. Ten days after inoculation, necrosis or cankerous lesions were ob-

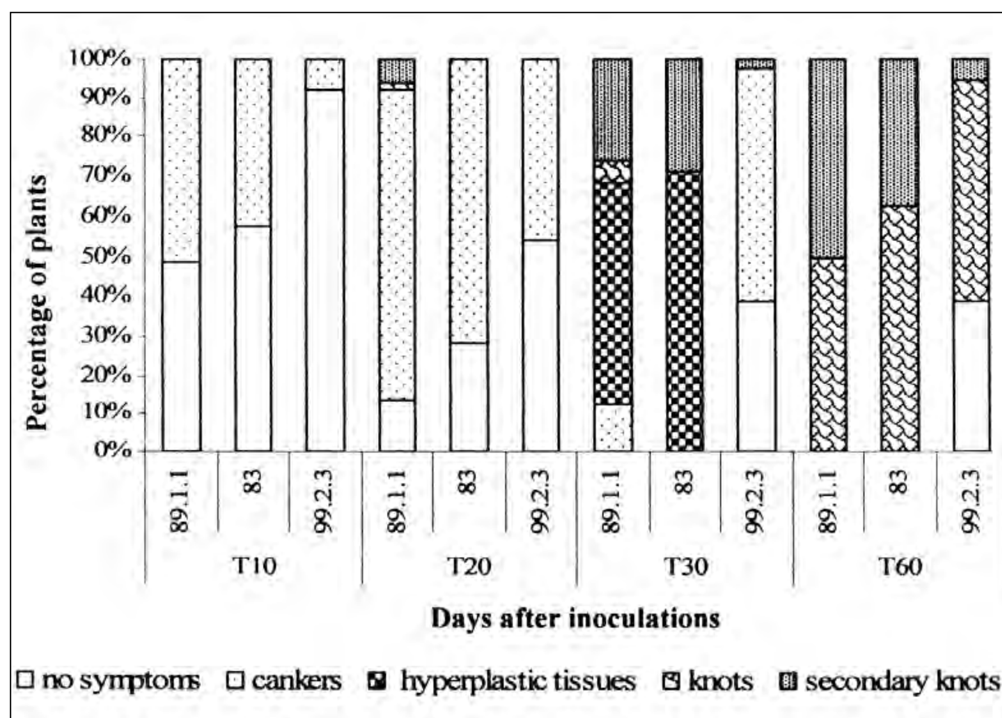

Fig. 1. Symptoms on different oleander accessions following inoculation with three strains of *P. savastanoi* pv. *nerii* (IPVCT 89.1.1, IPVCT 83 and IPVCT 99.2.3) of decreasing virulence. The 100% stacked bar shows the percentage contribution of plants that are healthy or with symptoms (described below) in a total of 90 plants (nine accessions, 10 plants per accession per strain) at four different intervals after inoculation. No symptoms: no reaction at inoculation point; Cankers: necrotic lesions at inoculation point; Hyperplastic tissue: new tissue formed at the edge of the lesion; Knots: localized gall-like overgrowth at inoculation point; Secondary knots: gall-like overgrowths at various places along the plant.

served at inoculation sites. Strains IPVCT89.1.1 and IPVCT83 induced cankerous lesions on 51% and 42% of inoculation sites respectively, but were seen only in 8% of sites inoculated with IPVCT99.2.3. Twenty days after inoculation, cankerous lesions had increased in size and hyperplasia was observed immediately around the lesions on plants inoculated with IPVCT89.1.1. Well-defined knots, sometimes associated with secondary knots, were first evident at inoculated sites on a few plants only, one month after inoculation. Two months after inoculation, localized knots and/or secondary knots were observed in 100% of plants inoculated with IPVCT89.1.1 and IPVCT83, but in only 61% plants inoculated with IPVCT99.2.3.

The reaction of the accessions was assessed using the DI at the last score registration (i.e. 4 months after inoculation) and the AUDPC.

DI ranged from 0 to 6.3, depending on accession and isolate (Table 1). The "Dark Salmon" oleander accession was the most susceptible with a mean DI (4.07) that was significantly higher than that of other accessions tested. On this accession inoculation with IPVCT89.1.1 or IPVCT83 led to death of shoot apices. "Dark Salmon", as well as "Petite Salmon" and "Salmon" flowered accessions (mean DI, 2.66 and 2.47), were highly susceptible showing severe deformation of stems, leaves and seed pods, and secondary knots on aerial parts. The accession "White" was the least susceptible (mean DI = 1.00). On this accession symptoms consisted of localized knots at the inoculation site, sometimes associated with secondary knots. Neither stem deformation, nor symptoms on inflorescence or seed pods were observed. The mean DI of the other accessions tested ranged between 1.27 and 1.80.

Symptoms induced by IPVCT89.1.1 (mean DI = 2.33) and IPVCT83 (mean DI = 2.59) did not significantly differ, but were significantly more severe than those induced by IPVCT99.2.3 (mean DI = 0.93). Inoculation with IPVCT99.2.3 did not lead to the disease in "Salmon" or in "White" accessions (Table 1).

P. savastanoi pv. *nerii* was always isolated from plants showing the above described symptoms.

AUDPC values were in agreement with the DI index results, where accessions "Dark Salmon", "Petite Salmon", and "Salmon" were again the most susceptible. "Dark Salmon" scored a very high AUDPC (401.40), as compared to the other accessions, when inoculated with strain IPVCT89.1.1, showing severe deformation and apex death (Table 2). Substantial differences in AUDPC also indicated an earlier appearance of symptoms. The progress curves with strain IPVCT89.1.1 show how the cultivars performed in the evaluation experiment (Fig. 2).

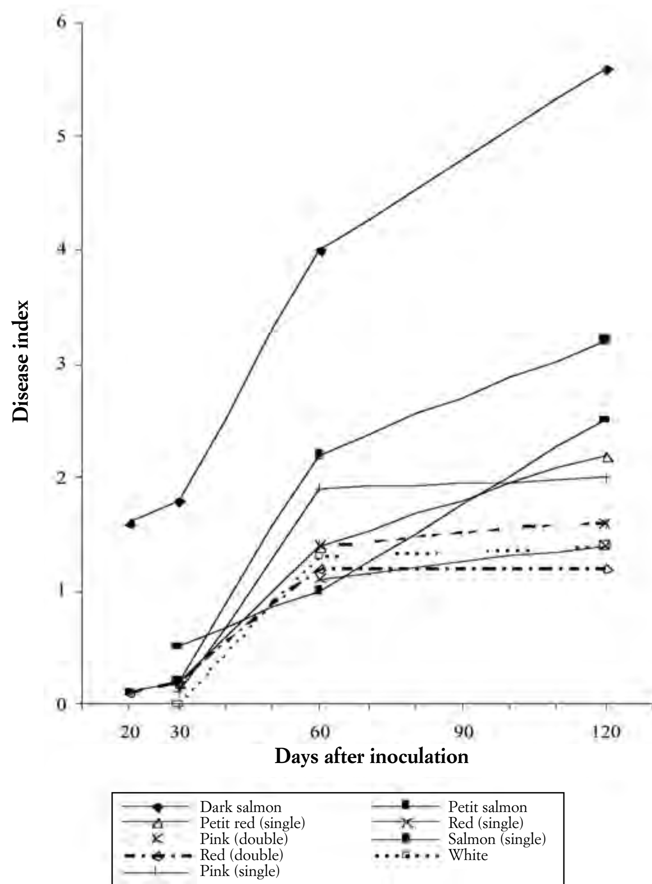

Fig. 2. Symptom severity progress in nine commercial oleander accessions inoculated with the most virulent strain of *P. s.* pv. *nerii*, IPVCT89.1.1., on a scale of 0-4 (0: no symptom; 1: localized knots at the inoculation point; 2: stem deformation; 3: stem deformation and apex death) according to primary symptoms induced 20, 30, 60 and 120 days after inoculation. The presence of secondary knots on leaves, flowers and pods was scored as one point per affected organ added to the class value.

Table 1. Disease index of nine accessions of *Nerium oleander* inoculated with three strains of *P. savastanoi* pv. *nerii* four months after inoculation.

Accessions	Disease index (DI)			
	IPVCT 89.1.1	IPVCT 83	IPVCT 99.2.3	Mean DI[a]
Dark Salmon	5.6	6.3	0.8	4.07 c
Petite Salmon	2.5	4.0	1.5	2.66 b
Salmon	3.2	4.2	0.0	2.47 b
Petite Red	2.2	2.0	1.2	1.80 ab
Pink (single)	2.0	1.8	1.2	1.67 ab
Red (double)	1.2	2.2	1.4	1.60 ab
Pink (double)	1.6	2.0	1.0	1.57 ab
Red (single)	1.4	1.0	1.4	1.27 ab
White	1.4	1.4	0.0	1.00 a
All	2.33 b	2.59 b	0.93 c	

[a] Numbers followed by same letters are not significantly different according to the Student-Keuls-Newman test, P=0.05.

Table 2. Area under disease progress curve (AUDPC) values for oleander accessions inoculated with three *P. savastanoi* strains.

Accessions	AUDPC		
	IPVCT 89.1.1	IPVCT 83	IPVCT 99.2.3
Dark Salmon	401.40	330.00	24.00
Petite Salmon	133.00	283.50	92.50
Salmon	205.05	263.75	0.00
Petite Red	136.70	121.75	84.55
Pink (single)	152.35	103.60	83.50
Red (double)	97.55	129.30	94.25
Pink (double)	114.50	112.25	32.10
Red (single)	94.25	81.70	89.50
White	103.75	94.25	0.00

The *in vitro* detached leaf assay. Initially explants from oleander stems and leaves were evaluated for development of an *in vitro* test following inoculation with ten *P. savastanoi* pv. *nerii* strains. One week after inoculation active bacterial multiplication was observed at the inoculation points both on stem and leaf pieces. In 99 stem pieces, *ca.* 0.5 cm in length, no knots developed at the inoculation points. Enlargements of the upper part of the stem were observed in 17 out of 66 stem pieces of 1.5 cm, but they were not induced by all strains. Symptoms on detached leaves were first observed 7 days after inoculation as small swellings or knots; one month after inoculation, depending on the strain inoculated, small knots, ranging from 1 to 5 mm in diameter, were observed at the inoculation point of 117 out of 300 leaf pieces (Table 3).

Given these results, it was decided to abandon the de-

Table 3. Disease Index (DI) and number of positive inoculations (PI) induced by *Pseudomonas savastanoi* pv. *nerii* strains on detached leaves.

Strains	Group of virulence[a]	PI	Disease index (DI)[b]
IPVCT 93.1.4	III	2	0.08 a
IPVCT 91.1.1	III	6	0.2 ab
IPVCT 99.2.3	III	7	0.23 ab
IPVCT 49	I	6	0.4 abc
IPVCT 83	II	11	0.5 abc
IPVCT 94.1.4	II	12	0.53 abc
IPVCT 89.1.1	I	14	0.53 abc
ITM 519	II	17	0.69 bc
IPVCT 100.1.3	II	16	0.76 c
IPVCT 87.1.1	I	26	1.73 d

[a] Classification of strains according to intensity of symptoms induced on stem of oleander (Pink single flower) following inoculation of a set of *P. savastanoi* pv. *nerii* strains (Bella *et al.*, 2001).

[b] Numbers followed by same letters are not significantly different according to the Student-Keuls-Newman test, P=0.05.

Table 4. Percentage of positive inoculations on detached leaves of three oleander accessions 7, 15 and 30 days after inoculation with *P. savastanoi* pv. *nerii*.

Strains	Accessions	Positive inoulations (%)		
		T7	T15	T30
IPVCT 87.1.1	Pink	100	100	100
	Dark Salmon	47	76	88
	White	31	69	85
IPVCT 93.1.4	Pink	100	100	100
	Dark Salmon	47	100	100
	White	5	22	22

Table 5. Disease Index (DI) calculated on the basis of size of knots (in mm), induced by *Pseudomonas. savastanoi* pv. *nerii* strains on detached leaves of three oleander accessions.

Accessions	Disease index (DI)[a]		
	T7	T15	T30
Pink	1.55 c	2.59 c	2.99 c
Dark Salmon	0.57 b	1.72 b	2.14 b
White	0.09 a	0.56 a	1.16 a
Strains			
IPVCT87.1.1	0.98 b	2.19 b	2.88 b
IPVCT 93.1.4	0.59 a	1.17 a	1.44 a

[a] Numbers in columns followed by same letters are not significantly different according to the Student-Newman-Keuls test, P=0.05.

tached stem assay and to choose the leaf explant assay, where symptoms were clearly identified as measurable knots. The strains tested showed different virulence with DI varying from 0.08 to 1.73 (Table 3). Three strains with a DI < 0.2 were significantly less virulent then strains with a DI > 0.76 (Table 3). The detached leaf assay was then applied to the most and least susceptible ("Dark Salmon" and "White") accessions of the *in* vivo test, together with the "Pink" (single flower) accession. Two *P. savastanoi* pv. *nerii* strains were chosen (IPVCT87.1.1 and IPVCT93.1.4) on the basis of their different ability to induce knots on leaves of the "Pink" accession in the first experiment and the groups of virulence as defined in Bella *et al*. (2001). Both inoculated strains, regardless of their virulence, induced knots on leaves, but the speed of the response of each accession to inoculation varied. Knot development was more rapid on "Dark Salmon" and "Pink" than on "White" (Table 4). One week after inoculation, IPVCT87.1.1 induced knots in 100%, 47% and 31% of leaves of "Pink", "Dark Salmon" and "White", respectively. The results for IPVCT93.1.4 were 100%, 47% and 5% respectively, for the same accessions (Table 4). The average knot size increased during the one-month observation period in all accessions inoculated with either of the strains (Table 5). The accession reacting most rapidly showed larger knots. The three accessions differed significantly in response to inoculation with *P. savastanoi* pv. *nerii* strains as early as seven days after inoculation. Observation at 15 and 30 days confirmed the different behaviour. *In vitro* inoculations confirmed that "Dark salmon" was more susceptible than "White" (Table 5). At each observation time knot size varied significantly in cultivars inoculated with IPVCT87.1.1 and IPVCT93.1.4 (Table 5), confirming the former strain as more virulent.

DISCUSSION

As commonly observed, the results showed that the nine oleander accessions tested responded differently to three isolates of *P. savastanoi* pv. *nerii* that differed in virulence, and no cultivar was resistant to all three isolates. Furthermore, the most ("Dark Salmon") and the least ("White") susceptible accession were clearly distinguished by our *in vitro* detached-leaf assay.

The difference in virulence of *P. savastanoi* pv. *nerii* strains was also confirmed, and the results of *in vivo* and *in vitro* inoculation supplied new detail on behaviour of strains: the strains previously separated in group I and II, now appeared to constitute a single virulence group, statistically differentiated from strains of group III.

Since inoculation of oleander plants led to a wide and complex range of symptoms, host-pathogen interactions were evaluated using an arbitrary 0-4 susceptibility scale originated on the basis of the description of the disease by Wilson and Magie (1964). They defined primary knots as those that develop at the initial infection sites, and secondary knots as those that may develop at various places along the invasion route, causing deformation of different plant organs. The appearance of secondary symptoms was registered as a value of plus one for each oleander organ affected. This scale appeared to reflect well the reaction of the accessions tested.

The different levels of susceptibility of the accessions to all the strains tested reveal a high degree of variability in the interaction between the host and pathogen. Part of this variability could be attributed to the different virulence of the strains (Bella *et al*., 2001) and it is thus recommended that a preliminary screening of the strains is performed before setting up evaluation trials. It is noteworthy that symptoms in the accession "White" were often limited to just knot formation at the inoculation points, or even no symptoms when infected with the mildest strain. On the contrary in the accession "Dark Salmon" severe deformation of stems and apex death were observed.

From the first description of the oleander knot disease caused by *P. savastanoi* pv. *nerii,* then named by Smith (1928) *P. tonelliana,* it was already noted that secondary infections occurred and that they were present

in the laticifers of the shoot cortex, and so not associated with the vascular system. Whether the extent of infection in laticifers is an expression of disease resistance remains to be investigated.

The "systemic" invasion of oleander represents a limit for *in vivo* screening, since no more than one plant per strain can be inoculated and therefore, an *in vitro* test based on inoculation of stems and leaf explants was evaluated.

Under experimental conditions, *in vitro* inoculation of detached leaves provided a simple and rapid method to test pathogenicity, infact evident hyperplastic tissue was already observed 7 days after inoculation, whereas in *in vivo* inoculation, only after 20-30 days. Thus the test seems suitable as a pathogenicity test, in particular when only laboratory conditions are available, but it is also very useful to rapidly screen a large number of bacterial strains or mutants. Furthermore, results obtained with the assay can confirm both differences in virulence of *P. savastanoi* pv. *nerii* strains and the different reactivity of oleander accessions assessed by *in vivo* inoculation.

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