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Evidence for a Correlation between Auxin Production and Host Plant Species among Strains of *Pseudomonas syringae* subsp. *savastanoi*

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Auxin production by 131 strains of *Pseudomonas syringae* subsp. *savastanoi* was investigated with the aim of looking for correlations among this characteristic and the origin of the strains, the types of symptoms, and the host plant. Most of the *P. syringae* subsp. *savastanoi* strains, except those isolated from ash, produced auxin and harbored *iaa* genes. Among ash strains, which were pathogenic only on ash, only 2 out of 33 were found to produce auxin and to harbor *iaa* genes.

Pseudomonas syringae subsp. *savastanoi* Janse 1982 (14) induces hyperplasia on oleander (*Nerium oleander* L.) and on several species of *Oleaceae*. This bacterium is commonly pathogenic on olive (*Olea europa* L.), ash (*Fraxinus excelsior* L.), privet (*Ligustrum japonicum* Thunb), jasmine (*Jasminum* sp.), forsythia (*Forsythia* sp.), and a Phillyrea sp. (3).

The symptoms of the disease vary as a function of the host plant (10a, 19). On olive, knots that develop on young stems and branches are colored green externally. After 6 months, the hyperplasic tissues darken and die. On young oleander shoots, the symptoms are very similar to those observed on olive. However, the knots and necrotic lesions along the secondary veins of the leaves are surrounded by a large chlorotic halo. In contrast, on ash the first symptoms appear as blisters on the bark. The symptoms further develop as wartlike formations but not as the typical knots observed on olive and on oleander (10a, 19).

The bacteria enter the tissues mainly through leaf scars and other injuries such as those caused by freezing temperatures, insect wounds, pruning wounds, etc. (4, 13, 24). Infection requires cambium activity and leads to the development of knots in a three-step process. These steps are (i) colonization of the plant tissues and formation of lysogenous cavities, (ii) proliferation of the plant cells around the infected cavities, and (iii) differentiation of xylem and possibly phloem cells (25).

Many studies suggest that knot formation on olive and oleander is correlated with a high concentration of indole-3-acetic acid (IAA) in infected tissues (1, 2, 18, 25). IAA production also seems to shorten the incubation period of the disease, whereas cytokinin production affects the enlargment of the knots (20). This was demonstrated by comparing the symptoms of the disease caused by a wild-type strain of *P. syringae* subsp. *savastanoi* with those caused by a mutant of the same strain deficient for IAA production (20). Some observations indicated that the production of IAA was associated with a better survival rate of *P. syringae* subsp. *savastanoi* (21). It has also been shown that bacteria which commonly inhabit the leaf surface of plants can produce IAA. However, the biological role of this function is not understood (23).

P. syringae subsp. *savastanoi* produces IAA from L-tryptophan via indoleacetamide as an intermediate. Two enzymes are involved in IAA synthesis. The first one, tryptophan 2-monooxygenase, catalyzes the conversion of L-tryptophan to indoleacetamide. The second one, indoleacetamide hydrolase, catalyzes the hydrolysis of indoleacetamide to IAA and ammonium (15, 16, 25). The genetic determinants for these enzymes were identified as two genes termed *iaaM* and *iaaH* (6). The locus encoding tryptophan 2-monooxygenase (*iaaM*) was cloned along with a large portion of gene *iaaH* in a pBR322 derivative to produce the 4.8-kb recombinant plasmid pLUC2 (6). The two *iaa* genes are plasmid borne in strains isolated from oleander (5) and chromosome borne in strains isolated from olive knots (7, 8).

Although molecular data on genes encoding IAA synthesis are available, little is known about the presence and expression of those genes in wild-type strains of *P. syringae* subsp. *savastanoi* isolated from various locations and hosts. The purpose of our study was to address this question with the aim of looking for correlations among IAA production, origin of the strains, types of symptoms, and host plant species.

A total of 143 strains were isolated at various locations and times, from six different hosts. Numbers of strains were as follows: 58 olive, 33 oleander, 39 ash, 6 privet, 4 phillyrea, and 3 jasmine. We first analyzed the taxonomic positions of these strains in comparison to those of pathovars of *P. syringae* by studying various phenotypical properties and levels of DNA-DNA hybridization (11). All strains but one were clustered in a single phenon. Clearly, *P. syringae* subsp. *savastanoi* constitutes a discrete DNA-relatedness group distinct from *P. syringae* subsp. *syringae* (11). In the present study, 131 strains from the single phenon were analyzed. These strains were isolated from olive (n = 53), oleander (n = 31), ash (n = 33), privet (n = 6), phillyrea (n = 3), and jasmine (n = 3) (Table 1).

The ability to produce IAA was investigated in vitro by using a colorimetric technique (12). Strains of *P. syringae* subsp. *savastanoi* were inoculated at ca. 2×10^5 cells ml^{-1}

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TABLE 1. Production of IAA by and presence of *iaaM* and *iaaH* genes in various strains of *P. syringae* subsp. *savastanoi*

Host plant or strain	Origin	No. of strains	Concn of auxin (μg/ml) in:								Hybridization signal[a]
			King B				King B plus 0.5 mM Trp				
			Avg	H[b]	L	SD	Avg	H	L	SD	
Ash	Algeria	2	NC[c]	1.5	2.25	NC	NC	10.7	16.8	NC	+++
	France, The Netherlands, United Kingdom, and United States	31	NC	ND[c]	ND	NC	NC	ND	ND	NC	−
Jasmine	Greece	3	NC	7.25	1.5	NC	NC	6.3	25.5	NC	+
Oleander	Algeria, France, Greece, Italy, The Netherlands, United States, and Yugoslavia	31	6.6	36.5	0.5	6.3	69.5	121.9	8.4	44.1	+ to +++
Olive	Algeria, France, Greece, Italy, Portugal, Syria, United States, and Yugoslavia	53	4.3	11.0	0.5	3.0	25.9	120.0	6.5	18.6	+ to +++
Phillyrea	Algeria	3	NC	5.75	1.25	NC	NC	17.3	7.8	NC	
Privet	Italy	6	15.6	24.5	13.0	4.4	76.5	92.5	37.0	20.8	+ to +++
Control strains											
P. syringae pv. syringae (lilac)	United Kingdom	1			4.5[d]				13.9[d]		−
p. syringae pv. tomato (tomato)	United Kingdom	1			NI[d]				13.9[d]		−
P. syringae pv. persicae (peach tree)	France	1			ND				ND		−
P. syringae pv. phaseolicola (French bean)	Canada	1			1.75[d]				13.3[d]		−
P. viridiflava (*Phaseolus* sp.)	Switzerland	1			0.5[d]				ND		−
E. coli[c]	NA	1			NI				NI		+

[a] Three levels of hybridization were recorded as follows: −, no signal; +, detectable signal; +++, strong signal.
[b] Abbreviations: H, highest value; L, lowest value.
[c] Abbreviations: NC, not calculated; ND, not detected; NI, not investigated; NA, not applicable.
[d] Only one strain of each pathovar has been studied; therefore, these values cannot be regarded as average values.

and grown for 48 h at 25°C with shaking in 5 ml of King B medium which did or did not contain 0.5 mM tryptophan (Trp). Bacteria were collected by centrifugation (15 min, 5,000 × *g*, 4°C), and the supernatant was saved for the assay. To assay IAA production, 1.5 ml of culture supernatant was added to 3 ml of modified Salkowski's reagent (12). The latter solution was made by adding 100 ml of concentrated sulfuric acid and 3 ml of 1.5 M $FeCl_3$ to 60 ml of water. The tubes were placed at room temperature for 30 min in the dark, and the optical densities at 530 nm were read with a Pye-Unicam Phillips spectrophotometer with IAA (Prolabo Chemical, Paris, France) as a standard (IAA was diluted in the growth media, i.e., King B or King B supplemented with 0.5 mM Trp). In the first set of experiments that were performed with regular King B medium, the detection limit was estimated as 0.5 μg/ml. However, when the experiments were performed with King B medium supplemented with 0.5 mM Trp, the blanks were higher and the detection limit shifted to 6 μg/ml. Unless otherwise specified, each experiment was done only once. The results are given in Table 1. Several conclusions can be drawn from these results. First, it appears that strains of *P. syringae* subsp. *savastanoi* did not all produce IAA, at least under these experimental conditions. Furthermore, a correlation between IAA production and the host plant was detected. Thus, strains isolated from jasmine, phillyrea, and oleander all produced detectable levels of IAA. The same was true for strains isolated from privet and olive. In contrast, out of 33 strains isolated from ash knots, only 2 were found to produce detectable levels of IAA. As previously reported, the concentration of IAA produced by the strains increased when these were grown in the presence of tryptophan (0.5 mM). Interestingly, the amounts of IAA synthesized by the bacteria varied as a function of the host plant from which the bacteria had been isolated. Highest, lowest, and average values of auxin concentrations in bacterial culture supernatants are given in Table 1. The values obtained for the three phillyrea strains and the three jasmine strains do not allow calculation of mean values for IAA production. The significance of these observations, if any, is not clear. The amount of auxin produced by the bacteria could be adapted to the sensitivity of the host plant to this hormone. In other words, olive cells might need less auxin (and possibly cytokinin) than oleander or privet cells to start dividing. To our knowledge, however, there are no experimental data to support this speculation.

In the second part of the study, the presence of *iaa* genes in strains of *P. syringae* subsp. *savastanoi* was investigated. In a first set of experiments, a colony hybridization technique was used to detect the presence of *iaaM* and *iaaH* genes. Bacteria were inoculated as light patches onto plates containing yeast extract-beef agar and grown overnight at 27°C. The next day, nylon transfer membranes (GeneScreen Plus; Dupont-NEN, Boston, Mass.) were placed on top of the plates in contact with the bacteria and were carefully lifted off the plates. Bacteria were lysed onto the membranes by using a standard alkaline treatment (17) essentially as indicated by the membrane manufacturer. Total DNA was cross-linked on the membranes, which were further hybridized with ^{32}P-labeled plasmid pLUC2 (6) according to standard procedures (17) and to the membrane manufacturer's instructions. Membranes were washed under stringent con-

ditions and were revealed by autoradiography. Results (Table 1) were scored as +++, +, and − as a function of the intensity of the hybridization signal (+++, very strong; +, detectable; −, no signal). A negative control strain did not yield any detectable hybridization signal, whereas the positive control strain did. Essentially, colony hybridization confirmed the results obtained by analyzing production of IAA in culture supernatants. The three jasmine strains, which produced IAA, harbored *iaa* genes as judged by the hybridization signal. The same was true for the 6 privet strains, for the 3 phillyrea strains, for the 31 oleander strains, and for the 53 olive strains. Among the 33 ash strains tested, only the 2 which produced IAA showed a strong hybridization signal with the probe (genes *iaaM* and *iaaH*). It is noteworthy that these two strains had been isolated in Algeria, a part of the world where ash was introduced only in the mid-19th century. However, oleander as well as olive are long-term indigenous plants in this country. Thus, these two strains may not be typical ash strains but rather oleander or olive pathogens which became virulent on ash.

To test the above hypothesis, we inoculated the ash strains, including the two Algerian isolates, on ash (*F. excelsior* L.), jasmine (*Jasminum nudiflorum*), oleander (*N. oleander* cv. Mme. Allen), and olive (*O. europa* cv. picholine) plants. Plants were wounded with a scalpel blade on five different young shoots; the ash plants were 1 year old. Bacteria were inoculated at ca. 2×10^6 CFU. The experiment was performed in spring (April). Plants were incubated under a field glasshouse, and the results were scored after 3 months. In our experimental conditions, only 31 out of 33 ash strains tested induced the disease on ash (94%). The two Algerian isolates provoked the disease on ash, with symptoms similar to those observed when plants were inoculated with the other ash pathogens. Interestingly, none of the ash strains induced symptoms of the disease on olive, oleander, or jasmine. Out of the 33 ash strains, only 15 were inoculated to phillyrea plants, and 18 were inoculated to forsythia plants. None were found to be virulent on these hosts. This clear-cut pathogenicity assay demonstrates that ash strains must be regarded as specific for this host. This result could be correlated with the nature of the symptoms of the disease, notably different on ash than on oleander or olive. As a consequence of the above results, the two Algerian strains (CFBP 2094 and CFBP 2176) producing IAA must be regarded as bona fide ash strains.

The origin of the *iaa* genes in the two Algerian ash strains is not known. However, they may originate from an oleander strain, since the *iaa* genes of this strain are located on extrachromosomal replicons (5). To investigate this hypothesis, we extracted (i) total (genomic) DNA and (ii) plasmid DNA from strains CFBP 2094 and CFBP 2176 and from another ash strain (CFBP 1663) which did not produce IAA and did not give any hybridization signal with the DNA probe pLUC2. Total DNA was obtained by using the technique of Dhaese et al. (9). Native plasmid DNA was extracted by using a modified alkaline lysis procedure (17), purified by isopycnic centrifugation on CsCl gradients, and separated in a 0.7% agarose gel. Total genomic DNA was submitted to restriction by endonuclease *Hin*dIII. Restriction fragments were separated in a 0.8% agarose gel. The DNA content of the two agarose gels was transferred onto nylon membranes and further hybridized with a purified 2.2-kb *Eco*RI-*Bam*HI fragment from pLUC2 (6) labeled with digoxygenin-modified nucleotide (Boehringer, Mannheim, Germany). This probe contained only part of the *iaaM* and *iaaH* genes. Clear hybridization signals were seen with genomic DNA of strains CFBP 2094 and CFBP 2176 but not with that of strain CFBP 1663, a result consistent with those obtained in colony hybridization (data not shown). However, except for that of the positive control lane which contained pLUC2 plasmid DNA, no signals were seen when the experiment was performed on CsCl-purified plasmid DNA (data not shown). The *iaaM* and *iaaH* genes in the two Algerian strains must therefore be located either on the chromosome of these strains or on very large plasmids which were not efficiently extracted. However, the technique used in this experiment routinely allows purification of megaplasmids such as the *Agrobacterium* pTi, pRi, and cryptic pAt plasmids. If megaplasmids exist in the two Algerian strains CFBP 2094 and CFBP 2176, they should be larger than the *Agrobacterium* plasmids, i.e., larger than 250 to 300 kb. However, known plasmids encoding IAA synthesis in *P. syringae* subsp. *savastanoi* were always smaller than 110 kb (8). Whatever the location of these genes in strains CFBP 2094 and CFBP 2176, their origin is unclear. From the above results, the presence of *iaa* genes in the two Algerian ash strains may result from plasmid transfer from an oleander strain. However, in this case the *iaa* genes must have subsequently become integrated into the bacterial chromosome of the Algerian isolates. Alternatively, a possible chromosome transfer (mobilization) from an olive strain or from any other *P. syringae* subsp. *savastanoi* strain with chromosomal *iaa* genes cannot be ruled out. However, since auxin synthesis is not absolutely required for pathogenicity on ash, the selective pressure accounting for such a phenomenon remains to be understood. As previously proposed, this trait (23) or a genetically linked function may improve the epiphytic survival of the pathogen.

Finally, a few comments on the control strains used in this work should be made. Very interestingly, several pathovars of *P. syringae* were found to produce detectable levels of a Salkowski-positive compound (Table 1). Such a compound, which is probably auxin (10, 22), was detected in culture supernatants of *P. syringae* pv. syringae (strain CFBP 1392), *P. syringae* pv. tomato (strain CFBP 2212), and *P. syringae* pv. phaseolicola (strain CFBP 1390). However, in colony hybridization assays, these strains failed to show any detectable hybridization signal with pLUC2 plasmid DNA. Our results are consistent with previously reported data (10, 22). Strain CFBP 1392, however, was isolated from lilac and not from pear or bean, as were those studied previously (22). Whether all of these strains do or do not harbor *iaa* genes related to *iaaM* and *iaaH* loci and whether they really do produce auxin remain unknown. Further studies are required to answer these questions.

The finding that ash strains did induce excrescence formation only on ash plants is of interest, since all *P. syringae* subsp. *savastanoi* strains are closely related. This specificity of ash strains cannot be attributed to the single lack of production of auxin, since two auxin-producing ash strains were isolated and shown to be pathogenic only on ash plants.

In addition to demonstrating the occurrence of correlations among the host plant, the symptoms of the disease, and auxin synthesis, this work has brought new data on the involvement of IAA in pathogenesis. Though this hormone seems to be involved in knot formation on olive and oleander, it appeared nonessential for disease development on ash.

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