Variability among Isolates of *Pseudomonas syringae* pv. *savastanoi* from the Phylloplane of the Olive

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(Received 25 October 1982)

Leaves of three or four different ages were taken from olive plants quarterly in 1974–1980. One thousand and fifty isolates of *Pseudomonas syringae* pv. *savastanoi* from the phylloplane were tested for virulence to the olive and subjected to numerical phenetic analysis using 60 unit characters. The data were analysed using unweighted average linkage (UPGMA) and single linkage clustering on the simple matching (S_{SM}) and pattern (S_P) coefficients. The isolates obtained from leaves of a given age at a given time of the year shared higher percentage similarity (S) values between themselves than with the others. Cluster composition was only marginally affected by different coefficients and methods of clustering. UPGMA analysis on the S_{SM} coefficient recovered 92% of the isolates in 10 major clusters at 75% S . Of the isolates from leaves of the same age collected at the same time of the year, 81–99% fell in the same cluster. Conversely, 91–97% of the isolates in five of the major clusters were from leaves of the same type. Of the isolates in the other major clusters, 95–98% were from two different sources but most of the isolates from leaves of one type segregated into discrete subclusters at 85–90% S . Hypothetical median organisms (HMOs) were constructed to represent all the isolates obtained from the leaves of each type each year. The resulting relationships between the HMOs confirmed those described above for the individual isolates.

INTRODUCTION

Pseudomonas syringae pv. *savastanoi* (Dye *et al.*, 1980; henceforth referred to as pv. *savastanoi*) causes an endemic disease of the olive (*Olea europaea*) known as ‘knot’ or ‘tubercle’. The primary symptoms consist of woody outgrowths that are most frequently seen on the young stems and on the branches and twigs. The leaves are rarely infected but they usually harbour pv. *savastanoi* on their surface.

Ercolani (1970) used a semi-selective medium to show that the density of pv. *savastanoi* on the phylloplane of the olive at three locations in 1969 did not increase with the severity of the disease on individual plants but was invariably greater in the spring and autumn (with a mean of 7.9×10^4 and 2.3×10^4 bacteria cm^{-2} , respectively) than in the winter and summer (6.6×10^3 and 5.8×10^2 bacteria cm^{-2} , respectively). Later work with a non-selective medium in 1973–1975 confirmed these results and showed that there were characteristic fluctuations in the populations of several other heterotrophic bacteria on the phylloplane (Ercolani, 1978).

Because the olive is an evergreen, the proportion of leaves of different ages on the trees changes markedly with time. In the south-east of Italy, the emergence of new leaves begins in the spring and parallels the activity of the cambium, being intense at the end of the spring and beginning of summer, slight in mid-summer and moderate in autumn (Armenise, 1950). The leaves persist on the branches for little over a year (Morettini, 1972). These circumstances prompted field work in 1973–1976 to determine whether seasonal fluctuation in the overall density of pv. *savastanoi* within the leaf canopy was affected by the changing distribution of the leaves among different age classes at different times of the year. This showed that (i) pv. *savastanoi* was invariably present on the leaves of any age at any time of the year, and (ii) the size

Table 1. *Meteorological data recorded during the month before each sampling*

Time of sampling		Average air temp. (°C)			Rainfall (mm)	Conditions of the sky (days)			Relative humidity (%) Mean
		Min.	Max.	Mean		Clear	Mixed	Cloudy	
1974	January	5.6	13.5	9.6	67.8	6	14	11	76
	April	8.4	15.8	12.1	118.6	4	13	13	71
	July	19.8	29.6	24.7	17.4	24	6	1	55
	October	10.3	19.2	14.8	98.4	5	18	8	67
1975	January	4.8	13.1	9.0	3.6	10	18	3	68
	April	9.9	17.4	13.7	12.0	13	10	7	66
	July	19.6	27.8	23.7	30.8	25	5	1	64
	October	13.0	21.5	17.3	38.8	11	17	3	68
1976	January	4.9	12.2	8.6	37.0	10	14	7	77
	April	8.8	17.4	13.1	63.6	5	8	17	74
	July	19.0	27.8	23.4	48.6	12	16	3	71
	October	13.9	21.7	17.8	143.2	8	13	10	71
1977	January	4.9	13.6	9.3	38.4	4	13	14	70
	April	8.7	18.5	13.6	10.0	9	10	11	58
	July	19.7	30.9	25.3	7.2	19	11	1	59
	October	11.6	21.4	16.5	2.0	14	14	3	65
1978	January	4.3	12.8	8.6	56.0	3	13	15	76
	April	9.1	16.8	13.0	40.4	1	9	20	73
	July	18.2	28.6	23.4	0.4	24	7	0	53
	October	11.5	19.9	15.7	100.2	7	11	13	76
1979	January	4.6	12.1	8.4	36.6	1	12	18	69
	April	8.1	16.9	12.5	54.2	5	10	15	74
	July	19.0	29.0	24.0	37.0	20	9	2	61
	October	12.8	22.4	17.6	50.8	5	15	11	75
1980	January	5.0	11.9	8.5	86.2	4	15	12	76
	April	7.4	16.0	11.7	11.9	8	12	10	64
	July	18.6	28.5	23.6	17.6	22	6	3	57
	October	12.5	21.5	17.0	44.8	6	15	10	73

of bacterial populations on individual leaves was under the influence of first-order interactions between the age of the leaves, the time of the year when these were formed, and the time of the year when they were examined (Ercolani, 1979).

Two explanations can account for the success of such bacteria as *pv. savastanoi* in colonizing the surface of leaves of different ages under a wide range of environmental conditions: either the bacteria are homogeneous but highly versatile in their nutritional and physiological requirements, or they exist in nature as heterogeneous populations that alternate on the phylloplane under the combined selective pressure of the host and the environment. The work described in this paper was intended to compare the characters of several isolates of *pv. savastanoi* obtained from olive leaves of different ages at different times of the year.

METHODS

Sampling and isolation. The field work was done in a grove of olives (cv. Coratina) at Bitonto (Bari) in S.E. Italy. A summary of prevailing meteorological conditions is presented in Table 1.

The plant material and methods of sampling were as described before (Ercolani, 1978, 1979) with the modifications indicated below. Several unfolding leaves were mapped on the trees at the end of March, the end of June and the end of September each year in 1973–1980. Five leaf samples, each with a total surface area of 1000 cm², were taken approximately 1, 4, 7, 10 and 13 months after mapping, starting in January, 1974, and continuing until October, 1980. Consequently, separate samples were made up of 4-, 7- and 10-month-old leaves at the end of January, 1-, 7-, 10- and 13-month-old leaves at the end of April, 1-, 4-, 10- and 13-month-old leaves at the end of July and 1-, 4-, 7- and 13-month-old leaves at the end of October each year in 1974–1980. A random sample of the

same size as those above was also taken from unmapped leaves at each time of sampling. Thus, leaf samples from 19 different sources were obtained each year. Fifteen samples consisted of leaves of one type and four of random leaves.

Numbers of pv. *savastanoi* were estimated on each sample by washing the leaves, plating the wash water on nutrient agar supplemented with 50 mg sucrose and 67 µg cycloheximide ml⁻¹, and incubating the plates 4 d at 26 °C. Fifty random colonies from those showing the typical morphology of pv. *savastanoi* and consisting of Gram-negative, oxidase-negative, arginine dihydrolase-negative, non-pectolytic, aerobic rods with polar flagella were purified, shown to be virulent for the olive, cv. Coratina, and stored on nutrient agar supplemented with 20 mg glycerol ml⁻¹ at 4 °C for up to 3 months.

Characterization of the isolates. Each isolate was examined for 20 phenotypic characters generated from two earlier studies (Bottalico & Ercolani, 1970; Ercolani, 1978). The tests were those that combined a probability of error (*p*; Sneath & Johnson, 1972) of 0.05 or less, with a separation figure (*C*; Gyllenberg, 1963) not significantly different from 0.5, when performed as described by Bottalico & Ercolani (1970) and Ercolani (1978). They were colonial characteristics (transparency, production of levan and diameter <2 mm); growth in broth at 38 °C, at pH 5.0, at pH 7.8 and with 3.0% (w/v) NaCl; use of malonate as a sole carbon source; acid from mannose, lactose, maltose, sucrose, *meso*-inositol and sorbitol; degradation of urea, tyrosine and Tween 40; resistance to oleandomycin at 15 µg ml⁻¹ and dihydrostreptomycin at 10 µg ml⁻¹; and pathogenicity (induction of a hypersensitive response in tobacco leaves with suspensions containing 10⁶ c.f.u. ml⁻¹).

Ten isolates from each leaf sample were selected at random from those characterized as above and further examined for 60 phenotypic characters (Table 2). The characters were the 60 best separators for 128 isolates of pv. *savastanoi* obtained from the surface of olive leaves of different ages at different times during a pilot study in 1973. Colonial characters were examined at 4 d on nutrient agar containing 1% (w/v) glucose. Nutrient broth containing 1% (w/v) glucose was inoculated with 10⁵ c.f.u. ml⁻¹ from an exponentially growing culture and incubated for 7 d to observe the occurrence and the characteristics of growth under different conditions. The utilization of substances as sole carbon sources was determined according to Sands *et al.* (1970). The degradation of Tween 40, tyrosine and urea was tested as described by Sierra (1957), Crosse & Garrett (1963) and Christensen (1946), respectively. The resistance to antibiotics contained in Sensi-Disks (BBL) was recorded at 24 h on plates of Antibiotic Medium 3 (Difco) solidified with 1.2% (w/v) agar and seeded with 4.5 × 10⁶ c.f.u. ml⁻¹ from an exponentially growing culture. The test for induction of a hypersensitive reaction in tobacco leaves was done according to Klement (1963). The incubation temperature was 26 °C for all tests except the hypersensitive reaction in tobacco leaves, for which it ranged between 20 and 26 °C. Duplication of three isolates throughout the study showed that observational error exceeded 5% in three tests (production of translucent colonies, formation of a pellicle in broth culture and degradation of tyrosine, for which it was 6.4, 5.9 and 7.1%, respectively) and remained below 5% in the others and averaged 3.2% over all the tests. These may be considered acceptable values (Sneath & Johnson, 1972).

Clustering of the isolates. The results of all tests were recorded in the '0' (negative) – '1' (positive) format and analysed on an IBM 370/158 computer using programs derived by S. Eberle from Wishart's (1969) Clustan 1.

The isolates examined for 60 characters were processed separately in two groups comprising the 1050 isolates from leaves of one type and the 280 isolates from random leaves, respectively. The isolates in each group were compared using the simple matching coefficient (*S_{SM}*) and the pattern coefficient (*S_P*) with both single linkage and unweighted average linkage (UPGMA) cluster analysis (Sneath & Sokal, 1973). The clusters and subclusters were defined at the 75 and 85% similarity (*S*) level, respectively. The vigour coefficient (Sneath, 1968) of each isolate was also measured and used for determining the mean vigour of each cluster. Clustering by the UPGMA analysis on the *S_{SM}* and *S_P* coefficients was repeated after all the isolates from the same sample had been replaced with a hypothetical median organism (HMO; Liston *et al.*, 1963) to represent them. This resulted in simplified *S*-matrices suitable for summarizing the relationships between the isolates obtained from different samples.

The data from the 20 tests on all the isolates were used for constructing one HMO for each sample. The 105 HMOs that represented the isolates from leaves of one type and the 28 HMOs for the isolates from random leaves were compared and clustered separately using the *S_{SM}* coefficient and UPGMA analysis.

RESULTS

*Clustering of the isolates by UPGMA analysis on the *S_{SM}* coefficient*

All but four of the 1050 isolates from leaves of one type were recovered in 10 major and 28 minor clusters (Fig. 1). Each of the major and minor clusters contained 60–134 and 2–5 isolates, respectively. The majority (81–99%) of the isolates from a given source were recovered in one of the major clusters while the others generated the minor clusters or merged with isolates from different sources into one of the other major clusters. Altogether, 92% of the isolates were thus

Fig. 1 (continued on opposite page). Simplified dendrogram of 1050 isolates of *Pseudomonas syringae* pv. *savastanoi* obtained from olive leaves of different ages at different times of the year in 1974–1980. The relationships shown are based on the S_{SM} coefficient and unweighted average linkage clustering (UPGMA). The isolates are identified by the age of the leaves from which they were obtained (01-, 04-, 07-, 10- and 13-month-old) and the time of the year when the leaves were that age (JN = January; AP = April; JL = July; OC = October).

Fig. 2. Simplified dendrogram of 280 isolates of *Pseudomonas syringae* pv. *savastanoi* obtained from random samples of olive leaves in 1974–1980. The relationships shown are based on the S_M coefficient and unweighted average linkage clustering (UPGMA). The isolates are identified by the time of the year when they were obtained (see legend to Fig. 1).

allocated to the major clusters. Between 91 and 97% of the isolates in clusters 5, 22, 26, 29 and 37 were obtained from one source, i.e. 1-month-old leaves in October, 10-month-old leaves in April, 7-month-old leaves in October, 4-month-old leaves in October and 10-month-old leaves in January, respectively. Clusters 2, 8, 12, 18 and 32 accommodated 83–99% of the 70 isolates obtained from each of two sources, i.e. 1- and 7-month-old leaves in April, 1- and 4-month-old leaves in July, 10- and 13-month-old leaves in July, 13-month-old leaves in April and October, and 4- and 7-month-old leaves in January, respectively. Most of the isolates from the same source segregated into discrete subclusters but 11–31% of the isolates from each source remained intermingled with others.

Of the 280 isolates from random samples, 243 were recovered in three major and 36 in seven minor clusters (Fig. 2). Two of the major clusters contained 70 and 97% of the isolates in January

and July, respectively; the third was divided into two subclusters, each containing 74 and 71 % of the isolates obtained in April and October, respectively.

Clustering of the isolates by UPGMA analysis on the S_p coefficient

The composition of the major clusters and their subclusters outlined in Fig. 1 remained virtually unchanged after removal of the vigour component. An exception was the isolates obtained from the younger leaves in April and July, the majority of which formed one cluster. Within this cluster, most of the isolates obtained from 1-month-old leaves in April and July formed one subcluster at 89% S and were joined by the majority of those obtained from 7-month-old leaves in April and 4-month-old leaves in July at 79 and 76% S , respectively. The resulting cluster, at one end of the S matrix, was flanked by the others in a somewhat different order from that in Fig. 1. This was paralleled by the different arrangement of the HMOs in the S_{SM} /UPGMA and S_p /UPGMA matrices (see below).

Use of the S_p instead of the S_{SM} coefficient had no visible effect on the composition or relative arrangement of the clusters of isolates from random leaves, except for the occasional shift of a few isolates from one cluster to an adjacent one.

Clustering of the isolates by single linkage analysis

The clustering technique did not appreciably modify the composition or the relative arrangement of the major clusters of isolates from random leaves.

With the isolates from leaves of one type, clustering by single linkage had different effects on the analyses based on the S_{SM} and S_p coefficients. When the former was used, most of the minor clusters defined by the UPGMA analysis were absorbed into one of the 10 major clusters, but these remained separated. Merging of the minor into the major clusters also occurred with the S_p coefficient. In addition, only three of the major clusters remained distinct. These were the clusters containing the majority of the isolates obtained from 4-month-old leaves in October, 10-month-old leaves in January and other leaves in January. Of the remaining clusters formed in the UPGMA analysis, those corresponding to the isolates from 7-month-old leaves in October and to the isolates from 10- and 13-month-old leaves in July were combined at 77% S , while all the others were grouped at 76% S into one aggregate corresponding to 554 individual isolates. When several UPGMA clusters merged together in the single linkage analysis, the corresponding isolates formed discrete subclusters. Use of single linkage analysis left the composition of the UPGMA subclusters almost unchanged.

Clustering of the HMOs

The results with the HMOs generated from data on 60 characters differed little from those obtained when 20 characters were used. The comments below apply to both sets of HMOs.

All the HMOs representing the isolates from leaves of one type were recovered in 10 clusters by S_{SM} /UPGMA analysis (Fig. 3). The HMOs representing the isolates from leaves of the same type always fell in the same cluster. Five clusters were each composed of seven HMOs representing the isolates from leaves of the same type. Each of the other five clusters contained 14 HMOs that represented the isolates from leaves of two types, but the HMOs corresponding to the isolates from leaves of one type segregated into discrete subclusters. The resulting dendrogram reproduced in simplified form the relationships already observed between the majority of individual isolates, without intermediates between the clusters (Figs 1 and 3). S_p /UPGMA analysis recovered the HMOs in nine clusters (Fig. 3). One large cluster contained the HMOs representing the isolates obtained from leaves of four types (1- and 7-month-old in April and 1- and 4-month-old in July) but the HMOs corresponding to the isolates from each source remained distinct at subcluster level. The other eight clusters maintained the same composition resulting from S_{SM} /UPGMA analysis but were arranged in different order.

The HMOs representing the isolates from random leaves fell in three clusters of identical composition by both S_{SM} and S_p /UPGMA analysis. The two peripheral clusters contained the HMOs for the isolates of January and July, respectively. The central cluster accommodated two subclusters corresponding to the HMOs for the isolates of April and October, as noted before with the individual isolates.

Fig. 3. Simplified dendrograms of the hypothetical median organisms (HMOs) representing the isolates of *Pseudomonas syringae* pv. *savastanoi* obtained from olive leaves of different ages at different times of the year in 1974–1980. The relationships shown are based on unweighted average linkage clustering (UPGMA) with the S_{SM} coefficient (above) and the S_P coefficient (below). The HMOs are identified by the age of the leaves from which the corresponding isolates were obtained and the time of the year when the leaves were that age (see legend to Fig. 1).

The similarity between the HMOs was not obviously correlated with the calendar year in which the corresponding isolates were obtained. Each cluster or subcluster defined at 85% *S* contained one to three HMOs with a higher mean resemblance to all other HMOs in the same group (Fig. 4). Such HMOs, however, were not generated with significantly higher frequency during any particular year.

Characterization of the major clusters

Table 2 shows the distribution of test responses to the 10 major clusters/subclusters illustrated in Fig. 1. These were the groups formed by S_{SM} /UPGMA analysis with the isolates from leaves of one type. No single test was cluster specific or suitable for discriminating between all the clusters. All the tests, however, proved useful for characterizing at least some of the clusters. The characters reported in Table 2 for clusters/subclusters 12B, 18A, 18B and 37 applied also to the four major groups of isolates from random leaves, i.e., 1A, 4A, 4B and 10, respectively (Fig. 2). Each of these groups contained the majority of the isolates obtained from random leaves in July, April, October and January.

The mean vigour of the clusters/subclusters in Table 2 ranged from 0.513 to 0.658 but was not significantly correlated with the age of the leaves from which the isolates were obtained or with the time of the year when the leaves were that age.

DISCUSSION

The characterization of microbial populations in natural habitats has been attempted by several authors with different methods, including numerical taxonomy (Oliver & Colwell, 1974; Goodfellow *et al.*, 1976), numerical profiles (Griffiths & Lovitt, 1980), principal component and factor analysis (Rosswall & Kvillner, 1978), reciprocal averaging (Houba & Remacle, 1980), time-series analysis (Fry *et al.*, 1981) and estimates of diversity by Shannon information index or Simberloff rarefaction method (Martin & Bianchi, 1980; Mills & Wassel, 1980). In this study, numerical phenetic analysis of epiphytic isolates of pv. *savastanoi* by single linkage or average linkage on the S_{SM} or S_P coefficients proved adequate to demonstrate a correlation between the source of the isolates and their phenotypic characters.

The populations of pv. *savastanoi* found on different samples of leaves of the same type over the years 1974–1980 shared a greater number of cultural and physiological characters with one another than with populations colonizing the leaves of a different type over the same years. The use of different similarity coefficients and clustering procedures did not affect the general validity of this conclusion but marginally modified the results of the analyses when the relationships between the isolates were defined at the 85% *S*-level. The well known chaining effect of single linkage clustering (Sneath & Sokal, 1973) became evident when the threshold for cluster formation was set at 75% *S*, especially if the S_P coefficient was used.

The correlation between source and phenotypic characters was confirmed with the isolates obtained from random leaves at three of the four sampling times each year. As a general rule the distribution of characters to the collections of isolates from random leaves could be anticipated from currently available data, i.e. the seasonal fluctuation in the proportion of leaves of different ages within the canopy of the olive (Morettini, 1972), the average density of pv. *savastanoi* on the leaves of different ages at different times of the year (Ercolani, 1979) and the composition of bacterial populations on the leaves of any one type (Fig. 1). On the assumption that the random samples taken during this work were representative of the true distribution of leaves, 74, 70 and 67% of the isolates in April, October and January could be expected to have the characters given in Table 2 for clusters/subclusters 18A, 18B and 37, respectively. The actual values were 74, 71 and 70%. In July, however, approximately 20, 26 and 54% of the isolates were expected with the characters of subclusters 8D, 12A and 12B (Table 2) but the actual values were <1, 19 and 74%, respectively. This finding can probably be explained by recurrent sampling errors in July, due perhaps to the spatial distribution of leaves of different ages at that time of the year. Additional data from 'random' samples in July should clarify this point.

Fig. 4. Sorted similarity matrix of the hypothetical median organisms (HMOs) representing the isolates of *Pseudomonas syringae* pv. *savastanoi* obtained from olive leaves of different ages at different times of the year in 1974–1980. The relationships shown are based on the S_{SM} coefficient and unweighted average linkage clustering (UPGMA). The HMOs are identified by the age of the leaves from which the corresponding isolates were obtained and the month and year when the isolations were made (see legend to Fig. 1; 74–80 = 1974–1980).

Table 2. *Characteristics of the major clusters/subclusters defined by UPGMA analysis on the S_{SM} coefficient*

Cluster/ subcluster number ... Source† ...	Character*	Percentage of isolates positive														
		2A 01AP	2B 07AP	5 01OC	8A 01JL	8D 04JL	12A 10JL	12B 13JL	18A 13AP	18B 13OC	22 10AP	26 07OC	29 04OC	32A 04JN	32B 07JN	37 10JN
Colonial characters:																
Minute (<2 mm)	Convex	88	87	12	73	79	94	90	89	85	81	15	26	84	93	13
	Entire	0	5	100	73	73	81	84	5	100	16	90	100	16	31	23
	Translucent	9	87	4	73	79	78	81	94	100	13	15	24	96	92	79
	Ringed	85	90	97	78	74	11	0	100	86	97	85	83	4	26	26
	Mucoid	86	85	13	71	77	94	88	92	88	89	13	24	82	90	15
	Fluorescent	9	7	3	0	0	86	95	21	93	100	87	89	20	13	26
Growth in broth:	Pellicle	100	100	85	63	82	3	45	100	100	27	25	98	2	95	98
	Sediment	81	85	97	67	82	93	100	8	2	0	15	26	2	19	84
	Growth at 38 °C	0	5	100	73	74	78	83	20	92	11	88	100	20	22	26
	Growth at pH 5	84	90	85	59	81	74	79	16	10	5	18	15	8	16	82
	Growth at pH 8	3	7	96	76	74	83	86	11	100	14	87	95	16	19	23
	Tolerance to NaCl 3.0% (w/v)	12	10	12	63	84	10	16	100	100	16	15	12	82	92	81
Sole carbon source (0.2% w/v):																
L-Arabinose	D-Fructose	88	93	100	65	85	93	100	20	0	0	15	29	4	29	85
	D-Galactose	19	10	0	2	0	94	98	19	83	95	88	82	23	16	29
	meso-Inositol	11	95	99	78	76	71	10	100	100	81	98	23	100	100	97
	Lactose	82	22	85	80	68	92	100	100	95	84	13	18	16	19	13
	Maltose	85	20	88	78	66	89	95	100	90	79	25	14	21	24	11
	Mannitol	90	2	6	80	10	90	0	15	84	93	13	90	0	15	15
	D-Mannose	96	8	7	78	12	90	6	0	76	92	10	90	0	12	20
	Raffinose	9	97	94	73	73	78	10	95	100	81	95	20	100	100	98
	D-Ribose	100	97	100	78	87	94	100	22	19	98	88	15	18	27	13
	Sorbitol	0	5	0	55	82	0	3	5	8	0	5	20	100	94	100
	Sucrose	18	22	79	59	23	10	0	22	93	100	12	95	16	22	26
	D-Xylose	100	100	82	71	85	81	86	27	20	86	100	15	100	100	21
	99	90	100	80	89	92	95	19	14	100	92	20	16	22	13	
	100	7	10	67	13	96	9	5	83	100	20	100	0	20	21	

Sole carbon source (0.1% w/v):

Acetate	14	20	81	65	26	6	0	19	85	100	15	97	14	21	26
<i>trans</i> -Aconitate	16	88	0	73	81	81	86	92	100	14	20	21	93	92	85
D- α -Alanine	97	100	84	69	82	79	84	17	14	83	93	9	100	100	10
DL-Asparagine	4	9	88	75	77	90	22	23	18	88	100	100	100	30	87
Betaine	6	10	93	75	83	84	18	27	23	90	100	100	100	22	100
Caproate	88	18	0	65	79	94	19	100	83	92	100	15	82	11	21
Citrate	9	90	97	73	71	78	10	92	98	84	98	18	96	100	100
Fumarate	82	100	81	8	32	92	88	89	88	83	15	80	82	92	81
Glutarate	96	95	85	61	87	0	10	98	95	14	18	100	4	98	95
DL-Glycerate	80	87	100	71	69	8	0	100	90	100	88	82	0	26	29
Heptanoate	100	95	90	73	29	81	86	23	12	87	80	98	91	5	92
L-Histidine	0	5	3	55	85	0	0	5	12	0	15	18	100	97	100
<i>p</i> -Hydroxybenzoate	100	100	85	65	81	76	83	25	17	89	95	15	98	100	8
DL- β -Hydroxybutyrate	16	20	82	53	21	3	0	21	88	98	18	100	20	27	21
Isoascorbate	16	10	18	61	81	4	10	100	100	13	20	11	84	92	82
D-Malate	4	10	84	73	82	81	16	16	10	87	100	98	93	19	90
Malonate	89	92	97	86	95	94	100	19	15	100	83	14	13	19	15
Pelargonate	100	100	82	57	85	0	19	100	98	11	15	100	5	100	97
L-Proline	84	100	100	12	31	92	88	94	93	83	13	80	86	95	82
Putrescine	3	8	0	57	87	0	0	5	17	3	15	23	98	92	94
D-Saccharate	97	92	87	67	26	83	88	11	17	84	82	100	100	23	95
Succinate	86	100	84	8	29	92	88	95	95	86	15	80	20	90	79
D-Tartrate	12	18	88	80	2	79	84	10	7	0	85	15	89	100	16
L-Tartrate	100	100	84	76	29	83	90	6	10	86	83	98	98	8	98
Uridine	20	5	15	63	82	8	14	100	97	19	2	8	80	90	82
Valerate	14	8	0	6	0	94	100	19	86	100	88	85	16	13	31
Degradation of:															
Tween 40	88	18	1	67	81	94	14	100	81	84	100	18	88	23	23
Tyrosine	18	23	85	90	2	83	88	20	0	0	85	18	88	100	19
Urea	88	93	100	78	74	4	0	100	85	100	90	85	2	23	29

Table 2—continued
Percentage of isolates positive

Cluster/ subcluster number ... Source† ...	2A 01AP	2B 07AP	5 01OC	8A 01JL	8D 04JL	12A 10JL	12B 13JL	18A 13AP	18B 13OC	22 10AP	26 07OC	29 04OC	32A 04JN	32B 07JN	37 10JN
Resistance to:															
Aminoisidine 10 µg	93	20	4	65	81	89	14	100	92	87	93	20	86	23	18
Novobiocin 15 µg	95	25	85	82	69	94	100	100	88	83	15	15	18	19	21
Oleandomycin 15 µg	91	90	16	71	77	92	86	86	81	84	12	26	82	92	18
Streptomycin 10 µg	15	20	90	86	2	79	83	20	2	5	83	12	86	100	16
Hypersensitivity in tobacco Dose 10 ⁶ c.f.u. ml ⁻¹	16	95	0	71	79	78	84	89	97	10	0	18	98	97	82
Vigour: mean‡ s.d.	0.549 0.047	0.532 0.039	0.607 0.049	0.642 0.042	0.604 0.060	0.658 0.041	0.539 0.050	0.521 0.028	0.636 0.057	0.618 0.058	0.527 0.049	0.517 0.055	0.543 0.056	0.518 0.049	0.513 0.048

* Additional tests explored during a pilot study in 1973 (see Methods) proved of little value as discriminators within *Pseudomonas syringae* pv. *savastanoi*. All the isolates possessed catalase and almost all grew at 4 °C, produced ammonia from peptone and utilized glucose, glycerol, *cis*-aconitate, γ -aminobutyrate, DL-aspartate, n-caprylate, D-gluconate, L-glutamate, L-glutamine, α -ketoglutarate, L-malate and mucate as sole carbon sources. Only a few isolates utilized one or more of the following substances as a sole carbon source: D-arabinose, D-fucose, L-fucose, methyl- α -D-glucoside, methyl- β -D-glucoside, L-sorbose, trehalose, 2-ketogluconate, dextrin, inulin, starch, salicin, adonitol, 2,3-butyleneglycol, dulcitol, erythritol, ethylene glycol, isobutanol, methanol, adipate, azelate, glycolate, isobutyrate, isovalerate, itaconate, maleate, mesaconate, oxalate, propionate, pectate, suberate, tannate, phenol, β -alanine, L-cysteine, L-cystine, glycine, L-isoleucine, L-lysine, D-methionine, L-methionine, D-threonine, L-threonine, D-tryptophan and L-tryptophan. All isolates gave a negative reaction for: growth at 40 °C or in the presence of 5.0% (w/v) NaCl; presence of phenylalanine deaminase; denitrification; production of indole or hydrogen sulphide from peptone; hydrolysis of arbutin, aesculin, gelatin or lecithin. Later tests showed that none of the isolates had ice-nucleating activity at -5 °C.

† The source of the majority of the isolates in each cluster/subcluster is indicated by the age of the leaves from which they were obtained (01-, 04-, 07-, 10- and 13-month-old) and the time of the year when the leaves were that age (JN = January, AP = April, JL = July, OC = October). The composition of each cluster/subcluster is shown in Fig. 1.

‡ Arithmetic mean of the vigour of all the isolates in each cluster/subcluster.

The isolates examined in this study did not differ appreciably in their growth rate. Some of the isolates with a given character in the positive state did differ in the time taken to respond in the pertinent test but the times of test reading were sufficiently delayed to minimize the influence of such differences on the S values. For this reason the results of the S_{SM} /UPGMA analysis gave perhaps a more accurate representation of the potential metabolic diversity of the isolates on the phylloplane. Only 60 characters were determined on each isolate in this study. However, the consistency between the results of different analyses and the invariance of most isolates of pv. *savastanoi* in 72 additional tests (Table 2) indicated that the observed pattern of relationships between the isolates is soundly based. The relative invariance of pv. *savastanoi* in the additional tests, most of which are useful discriminators among plant pathogenic fluorescent pseudomonads, also has a taxonomic implication. Since the 60 tests used in this study were selected to give maximum discrimination, the resulting S values were disproportionately low. Therefore caution is needed not to equate the major clusters in Fig. 1 with any formal taxon.

Earlier descriptions of pv. *savastanoi* agree on the most frequent state of several characters but not of others (Misaghi & Grogan, 1969; Sands *et al.*, 1970). This makes comparison with the present results difficult. The utilization of lactose, maltose or raffinose, in particular, by some of the epiphytic isolates is in contrast to the data of both Misaghi and Grogan (1969) and Sands *et al.* (1970). However, acid production was recorded by Dye (1956) from lactose and by Bottalico & Ercolani (1970) from all three sugars, with at least some of their isolates of pv. *savastanoi*. Janse (1982) found that 18 isolates of the bacterium causing excrescences on *Oleaceae* and *Nerium oleander* were sufficiently distinct from five other members of *P. syringae* to justify subspecies rank for them, as *P. syringae* subsp. *savastanoi*. However, Janse's differential biochemical tests failed to separate several epiphytic isolates of pv. *savastanoi* from any of 20 authentic cultures of *P. syringae* pvv. *syringae*, *lachrymans*, *morsprunorum*, *pisi* and *tomato* (G. L. Ercolani, unpublished observations). Therefore the name proposed by Dye *et al.* (1980) for the olive knot pathogen, i.e. *P. syringae* pv. *savastanoi*, was retained in this paper.

In principle, the phenetic diversity of phylloplane isolates of pv. *savastanoi* from different sources may serve as a useful tool in epidemiological studies but this approach requires judgement. Control experiments showed that the characters of the isolates obtained most frequently from leaves of a given age at a given time of the year differed in different groves where several olive cultivars were grown under varied conditions. The significance of individual characters in respect of the ability to colonize the phylloplane under a certain set of conditions has yet to be determined. Therefore, for the present the potential for epiphytic survival of a given phenotype can only be anticipated within the boundaries of the environmental niche where it was found originally.

While this work confirmed that pv. *savastanoi* undergoes seasonal fluctuation on the phylloplane of the olive (Ercolani, 1970, 1978, 1979), it also poses additional questions about the composition of bacterial populations colonizing the surface of leaves of different ages at different times of the year. Several plasmids have been identified in Italian isolates of pv. *savastanoi* (Comai *et al.*, 1982) but their role in the regulation of the phenotypic characters considered in this study is not known. Experiments to be described elsewhere showed that spray-inoculation of the leaves with an appropriate mixture of isolates reproduced the alternating prevalence of different populations of pv. *savastanoi* on the phylloplane as the leaves aged. Then, if the same isolates were inoculated separately, the initial rate of growth of each of them on the phylloplane was invariably greatest under such conditions as could be recorded during the period when that isolate was prevalent on the leaves inoculated with the mixture. The experiments showed also that the phenotypic characters of the isolates remained stable during growth on the phylloplane. This suggests that alternation of heterogeneous populations rather than recurrent modification of a homogeneous population was responsible for the observed phenotypic fluctuation of pv. *savastanoi* in nature.

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