Pseudomonas savastanoi and Other Bacteria Colonizing the Surface of Olive Leaves in the Field

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Aerobic, heterotrophic, mesophilic bacteria were isolated from olive leaves in January, April, July and October in each of three consecutive years. Phenetic data on the isolates and marker strains were collected and analysed using numerical taxonomic methods: 1744 of the 1789 isolates were recovered in phenons that were equated with *Pseudomonas savastanoi* (67.86% of the isolates), *Erwinia herbicola* (8.50%), *Bacillus megaterium* (4.02%), *Micrococcus luteus* (3.63%), the *Xanthomonas campestris* group (3.35%), *Arthrobacter globiformis* (2.07%), *Lactobacillus plantarum* (1.45%), *Klebsiella pneumoniae* (1.40%), *Serratia marcescens* (1.34%), *Acetobacter aceti* (1.23%), *Leuconostoc dextranicum* (1.12%), *Pseudomonas fluorescens* (1.06%), *Bacillus subtilis* (0.34%) and *Pseudomonas delafieldii* (0.11%). There were characteristic seasonal fluctuations in the populations of most of the bacteria. The abundance of *P. savastanoi* on healthy leaves in April and October supports earlier suggestions that the phylloplane of the host may be an important source of readily available inoculum in the epidemiology of olive knot disease.

INTRODUCTION

Pseudomonas savastanoi (Dye *et al.*, 1975) causes the endemic disease olive knot or tubercle, which occurs in most regions of the world where *Olea europaea* (olive) is grown. The disease gets its name from the woody outgrowths that are most frequently found on young stems and on branches and twigs where they originate as wound infections (Smith, 1920). The organism causes a similar disease on other plants, including *Fraxinus excelsior* (ash), *Nerium oleander* (oleander) and *Ligustrum japonicum* (Japanese privet).

In south-east Italy, where the olive is grown extensively, the incidence and intensity of the disease increase markedly following damage to plants, especially by freezing (Ciccarone, 1964), during the spring and autumn when the cambium is active (Armenise, 1950). These observations suggest that the factors determining the prevalence of the disease may include seasonal variation in the size of the minimal effective dose, the amount of available inoculum and the extent and time of wounding. Ercolani (1971) found that the number of *P. savastanoi* isolates from washings of symptomless leaves, collected at three locations in south-east Italy in 1969, was not correlated with the severity of the disease in individual plants but was greater in April and November (mean 7.9×10^4 and 2.3×10^4 viable cells cm^{-2} , respectively) than in January and August (6.6×10^3 and 5.8×10^2 viable cells cm^{-2} , respectively). These results, obtained using a partially selective medium for *P. savastanoi*, suggest that *P. savastanoi* populations on olive leaves may be an important source of readily available inoculum in the spring and autumn but they throw little light on the ecological relationships of *P. savastanoi* in the phylloplane microflora at different times of the year. It is, therefore, difficult to determine the relative significance of the healthy plant, the diseased tissue, the epiphytic microflora and the weather in determining the size of *P. savastanoi* populations available for infection.

Table 1. *Meteorological data recorded at sampling site*

Date of sampling	Average air temp. (°C) during the 10 d before sampling			Rainfall (mm d ⁻¹) during the 7 d before sampling on day 0						
	Min.	Max.	Mean	7	6	5	4	3	2	1
1973 Jan. 22	4.6	12.0	8.1	0.0	1.1	0.0	0.7	4.4	0.0	0.0
Apr. 18	5.5	14.2	9.8	0.0	1.2	14.8	5.6	0.0	0.0	0.0
Jul. 23	21.1	31.9	26.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Oct. 22	12.0	17.8	15.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1974 Jan. 23	4.2	11.6	7.8	0.0	2.4	2.9	2.2	0.0	0.0	0.0
Apr. 22	6.9	14.1	10.1	2.4	25.1	14.1	3.9	3.8	0.0	0.0
Jul. 24	20.4	33.8	27.4	0.0	0.0	0.0	15.5	9.0	0.0	0.0
Oct. 24	10.9	19.2	15.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0
1975 Jan. 20	3.8	13.1	8.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
Apr. 21	9.5	16.7	12.9	0.0	0.0	2.0	0.0	0.0	0.0	0.0
Jul. 21	22.4	30.3	26.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Oct. 26	13.1	21.1	17.4	0.8	0.0	0.3	0.0	13.1	0.0	0.0

The primary aim of the present work was to follow the seasonal fluctuations in bacterial populations on the phylloplane of olive using an isolation procedure designed to select aerobic, heterotrophic, mesophilic bacteria.

METHODS

Sampling and isolation procedures. A group of 10 trees, centrally located in an olive grove, cv. Coratina, at Bitonto, Bari, Italy, was sampled quarterly from January 1973 to October 1975. Meteorological data were also recorded during this period (Table 1). On each sampling occasion, 10 clean healthy leaves were collected from each of four branches on each tree, bulked and taken immediately to the laboratory. Four random batches of leaves with a surface area of 1500 cm² (adaxial plus abaxial), determined with a type AAM-5 area meter (Hayashi Denko, Tokyo, Japan), were washed separately in 1.5 l of $\frac{1}{4}$ -strength Ringer solution in a 3 l flask on a reciprocal shaker (200 strokes of 3 cm min⁻¹) in the dark for 2 h at room temperature. Controls (Crosse, 1959), using yeast tryptone glucose extract agar [YTGEA: Difco tryptone glucose extract agar, 2.4 % (w/v); Difco yeast extract, 0.1 % (w/v); glucose, 0.4 % (w/v)] supplemented with 67 µg cycloheximide ml⁻¹ as the plating medium, indicated that over 90 % of the phylloplane bacteria passed into suspension and that there was no error due to bacteria multiplying in the washing fluid. These findings were supported by plating 10-fold serial dilutions of homogenates of washed and unwashed leaves on the same medium as above. There was also evidence that virtually no bacteria passed into suspension from inside the leaves (Ercolani, 1976). Cycloheximide did not significantly affect the recovery of *P. savastanoi* and *Erwinia herbicola* from the leaves but its effect on other bacterial taxa could not be determined as fungal contamination impaired the precision of the controls.

After washing, 1.0 ml samples of a 10⁻⁸ dilution, pooled from the supernatant liquid, were pipetted into three Petri dishes and 12 ml of molten (48 °C) YTGEA plus cycloheximide was added to each. The poured plates were surface-dried at 38 °C for approximately 30 min and then incubated at 28 °C for 5 d. Colonies visible to the naked eye were purified by streaking twice on YTGEA and incubating the plates at 28 °C. Isolates obtained at the four sampling times during the first, second and third years of work were designated collectively as sets 1:1 to 1:4, 2:1 to 2:4 and 3:1 to 3:4, respectively. Altogether, there were 1789 isolates: 70 in set 1:1, 346 in 1:2, 56 in 1:3, 104 in 1:4, 72 in 2:1, 281 in 2:2, 68 in 2:3, 130 in 2:4, 59 in 3:1, 418 in 3:2, 73 in 3:3 and 112 in 3:4.

The isolates were examined with the reference cultures listed in Table 2. Cultures R1 to R6 were included in the study from the beginning; they represented phylloplane bacteria encountered most frequently in preliminary isolation trials in several olive groves in October 1972. Additional reference cultures were added later to represent bacterial taxa identified during the present study.

Characterization of bacterial isolates. As soon as all of the isolates from a set had been purified, they were screened, together with reference cultures R1 to R6, for the 210 phenotypic characters listed in Table 3. Characters 1 to 58, 61 to 105, 145 to 163, 165 to 176, 180, 182, 185 to 188, 190 to 195, 199 to 201, 203, 204 and 206 to 208 were determined according to Colwell & Wiebe (1970) with a number of modifications. Thus,

Table 2. Reference cultures

Taxon	Culture source/no.	Designation
<i>Acetobacter aceti</i>	NCIB 8621	R7
<i>Arthrobacter globiformis</i>	ATCC 8010	R8
<i>Bacillus megaterium</i>	ATCC 14581	R1
<i>Bacillus subtilis</i>	ATCC 6051	R9
<i>Erwinia herbicola</i>	ATCC 12287	R2
<i>Flavobacterium capsulatum</i>	ATCC 14666	R3
<i>Klebsiella pneumoniae</i>	ATCC 13883	R10
<i>Lactobacillus plantarum</i>	ATCC 14917	R4
<i>Leuconostoc dextranicum</i>	ATCC 19255	R11
<i>Micrococcus luteus</i>	ATCC 4698	R12
<i>Pseudomonas delafieldii</i>	ATCC 17505	R13
<i>Pseudomonas fluorescens</i>	ATCC 13525	R14
<i>Pseudomonas savastanoi</i>	NCPFB 2327	R5
<i>Serratia marcescens</i>	ATCC 13880	R15
<i>Xanthomonas campestris</i>	NCPFB 528	R6

sodium chloride was omitted from basal media; both square and round cell ends were recorded as blunt ends; luminosity was not determined; and the results of tests with antibiotics were scored in terms of resistance rather than sensitivity.

In the tests for consistency of colonies, mucous and butyrous growth were recorded after 4 d on nutrient agar containing sucrose (5 %, w/v). The abilities of isolates to grow in nutrient broth containing sodium chloride (2, 5 and 10 %, w/v), nutrient agar containing crystal violet ($4 \mu\text{g ml}^{-1}$), azide dextrose broth (Difco) and lauryl tryptose broth (Difco) were recorded after 7 d. The utilization of substrates as sole carbon and energy sources was determined using the basal medium and method of Stanier *et al.* (1966). Phenylalanine deaminase was detected according to Ewing (1962). The hydrolysis of aesculin and arbutin was tested in liquid (Sneath, 1956) and solid (Crosse & Garrett, 1963) media, respectively. Haemolysis was observed after 3 d on nutrient agar containing sodium chloride (0.85 %, w/v) and defibrinated horse blood (5 %, v/v). The degradation of pectate and cellulose were determined on modified pectate medium (Cuppels & Kelman, 1974) after 3 d and on cellulose mineral salts medium (Skerman, 1967) after 30 d, respectively. Heart infusion agar (Difco) containing egg yolk emulsion (10 %, v/v; Oxoid) was used for determining degradation of lecithin after 5 d, and urea agar (Difco) for detecting urea decomposition after 2 d. The tests for gluconate oxidation and tetrazolium reduction were performed according to Haynes (1951) and Whittenbury (1965), respectively. Soft rotting of potato slices (Lelliott *et al.*, 1966) and induction of a hypersensitive reaction (HR) in tobacco leaves (Klement, 1963) were recorded after 2 d.

All tests were done at 28 °C and the results were coded on standard IBM cards in the '0' (negative), '1' (positive) format for analysis using an IBM 370/125 computer.

Computer analysis and identification of clusters. The following procedure, derived from Oliver & Colwell (1974), was used for comparing the isolates within and between sets and for identifying the clusters.

(i) The data were analysed using the Jaccard coefficient (S_j ; Sneath, 1957) and sorted similarity matrices were obtained for each set using single linkage cluster analysis (Sokal & Sneath, 1963). Clusters formed at or above the 70 % similarity level (S level) were assigned progressive arabic numerals within each set.

(ii) The mean similarity values within and between clusters defined under (i) were calculated.

(iii) A hypothetical median organism (HMO) was generated for each cluster by comparing the phenotypic data of all isolates within the cluster and coding the HMO '0', '1' and '3' for those characters that were shared by 0 to 33, 67 to 100 and 34 to 66 % of the isolates in the cluster, respectively. The HMOs were assigned identification numbers corresponding to the set and cluster number from which they were derived, and their phenotypic data were entered in an $n \times t$ table as before, except that characters coded '3' were ignored and consequently the number of coded characters involved in making comparisons ranged from 171 to 210. The data were analysed using the S_j coefficient and the single linkage cluster analysis technique. Clusters of HMOs formed at or above the 70 % S level were defined, and the mean similarity values within and between clusters were calculated as before. For each of the clusters containing HMOs a bar graph was prepared with the HMO identification number as the abscissa and the number of isolates in the cluster from which each HMO was generated as the ordinate.

(iv) Isolates previously assigned to clusters within the sets were rearranged into aggregate clusters which were given a progressive roman numeral and delimited to include only those isolates that had generated HMOs belonging to the same cluster. Within each aggregate cluster, the isolate with the highest average S value with the HMOs generated from that aggregate was selected as the type isolate. Type isolates were

Table 3. *Characters examined**

Morphology and staining		Mode of utilization of carbon sources	
1 to 5.	Length	145 to 148.	Glucose, oxidation-fermentation
6 to 9.	Width	149 to 150.	Ribose, acid/gas from
10 to 19.	Shape	151 to 152.	Galactose, acid/gas from
20 to 24.	Endospore formation	153 to 154.	Lactose, acid/gas from
25 to 27.	Gram stain	155 to 156.	Sucrose, acid/gas from
28 to 33.	Flagella and motility	157 to 158.	Salicin, acid/gas from
34 to 35.	Inclusions	159 to 160.	Mannitol, acid/gas from
36 to 43.	Cell arrangement	Nitrogen metabolism	
Colonial characteristics		161.	Arginine dihydrolase
44 to 46.	Size	162.	Lysine decarboxylase
47 to 50.	Elevation	163.	Ornithine decarboxylase
51 to 55.	Edge	164.	Phenylalanine deaminase
56 to 58.	Density	165.	Peptone, NH ₃ from
59 to 60.	Consistency	166 to 174.	Growth on media <i>a</i> to <i>i</i>
61 to 76.	Pigment formation	175 to 176.	Nitrate, nitrite/gas from
Growth in broth		Degradation of miscellaneous substrates	
77 to 83.	Type of growth	177.	Aesculin
84 to 94.	Temperature range	178.	Arbutin
95 to 105.	pH range	179.	Blood
106 to 108.	Osmotic range	180.	Casein
Sole carbon sources		181.	Cellulose
109.	Arabinose	182.	Gelatin
110.	Ribose	183.	Lecithin
111.	Xylose	184.	Pectate
112.	Fructose	185.	Starch
113.	Galactose	186.	Tween 20
114.	Glucose	187.	Tweeer 40
115.	Mannose	188.	Tween 80
116.	Lactose	189.	Urea
117.	Sucrose	Antibiotic resistance	
118.	Raffinose	190.	Chloromycetin, 30 µg
119.	Methyl α-D-glucoside	191.	Colimycin, 10 µg
120.	Methyl β-D-glucoside	192.	Dihydrostreptomycin, 10 µg
121.	Ethanol	193.	Oleandomycin, 15 µg
122.	Glycerol	194.	Penicillin, 10 units
123.	Mannitol	195.	Tetracycline, 30 µg
124.	meso-Inositol	Tolerance to selective agents	
125.	Sodium acetate	196.	Crystal violet
126.	Sodium <i>p</i> -hydroxybenzoate	197.	Sodium azide
127.	Sodium citrate	198.	Sodium lauryl sulphate
128.	Sodium formate	Miscellaneous characters	
129.	Sodium fumarate	199.	Catalase activity
130.	Sodium 2-ketogluconate	200.	Oxidase activity
131.	Sodium 2-ketoglutarate	201.	Phosphatase activity
132.	Sodium DL-lactate	202.	Gluconate oxidation
133.	Sodium D(+) -malate	203.	Acetoin production
134.	Sodium maleate	204.	Methyl red, acid colour
135.	Sodium malonate	205.	Tetrazolium reduction
136.	Sodium oxalate	206.	Indole production
137.	Sodium propionate	207.	Cysteine .HCl, H ₂ S from
138.	Sodium succinate	208.	Sodium thiosulphate, H ₂ S from
139.	Sodium D-tartrate	209.	Potato soft rot
140.	Sodium L-tartrate	210.	Hypersensitivity in tobacco
141.	DL-α-Alanine		
142.	L-Glutamic acid		
143.	DL-Methionine		
144.	L-Proline		

* For details of tests, see Methods.

Table 4. *Distribution and identification of isolates from olive leaves*

Set and cluster no.*	No. of isolates in cluster	Aggregate cluster no.†	Identification of type isolate in aggregate cluster and its %S range with other isolates in the cluster	Reference culture‡ and %S range with isolates in the cluster
1:1, 1	2	I	<i>Pseudomonas delafieldii</i> (100)	R13 (79)
1:1, 2	23	II	<i>Pseudomonas savastanoi</i> (77-97)	R5 (81-94)
1:1, 3	5	III	<i>Pseudomonas fluorescens</i> (93-98)	R14 (83-88)
1:1, 4	6	IV	<i>Xanthomonas campestris</i> group (80-89)	R6 (80-90)
1:1, 5	8	V	<i>Erwinia herbicola</i> (89-98)	R2 (88-95)
1:1, 6	5	VI	<i>Klebsiella pneumoniae</i> (85-92)	R10 (80-88)
1:1, 7	4	VII	<i>Bacillus subtilis</i> (92-99)	R9 (75-84)
1:1, 8	5	VIII	<i>Bacillus megaterium</i> (86-95)	R1 (83-91)
1:2, 1	330	II	<i>P. savastanoi</i> (76-100)	R5 (80-92)
1:2, 2	5	V	<i>E. herbicola</i> (88-98)	R2 (87-96)
1:2, 3	8	VIII	<i>B. megaterium</i> (82-98)	R1 (82-89)
1:2, 4	3	IX	<i>Micrococcus luteus</i> (93-99)	R12 (78-86)
1:3, 1	2	II	<i>P. savastanoi</i> (99)	R5 (83-84)
1:3, 2	8	IV	<i>X. campestris</i> group (79-90)	R6 (76-88)
1:3, 3	6	V	<i>E. herbicola</i> (84-97)	R2 (83-95)
1:3, 4	8	X	<i>Serratia marcescens</i> (86-98)	R15 (80-83)
1:3, 5	7	VIII	<i>B. megaterium</i> (81-96)	R1 (84-95)
1:3, 6	4	XI	<i>Leuconostoc dextranicum</i> (89-98)	R11 (80-91)
1:3, 7	2	XII	<i>Lactobacillus plantarum</i> (98)	R4 (82-84)
1:3, 8	4	XIII	<i>Arthrobacter globiformis</i> (94-99)	R8 (80-83)
1:3, 9	13	IX	<i>M. luteus</i> (86-98)	R12 (78-94)
1:4, 1	48	II	<i>P. savastanoi</i> (79-97)	R5 (79-87)
1:4, 2	8	IV	<i>X. campestris</i> group (80-92)	R6 (75-84)
1:4, 3	3	XIV	<i>Acetobacter aceti</i> (96-98)	R7 (84-96)
1:4, 4	26	V	<i>E. herbicola</i> (81-100)	R2 (76-92)
1:4, 5	2	VIII	<i>B. megaterium</i> (96)	R1 (81-83)
1:4, 6	6	XII	<i>La. plantarum</i> (82-97)	R4 (80-90)
1:4, 7	8	XIII	<i>Ar. globiformis</i> (96-100)	R8 (76-89)
1:4, 8	2	IX	<i>M. luteus</i> (100)	R12 (76)
2:1, 1	15	II	<i>P. savastanoi</i> (80-98)	R5 (81-93)
2:1, 2	7	III	<i>P. fluorescens</i> (94-98)	R14 (81-90)
2:1, 3	9	IV	<i>X. campestris</i> group (80-91)	R6 (78-90)
2:1, 4	12	V	<i>E. herbicola</i> (85-100)	R2 (80-94)
2:1, 5	10	VI	<i>K. pneumoniae</i> (85-96)	R10 (76-85)
2:1, 6	7	VIII	<i>B. megaterium</i> (83-100)	R1 (81-90)
2:1, 7	3	IX	<i>M. luteus</i> (97-100)	R12 (78-90)
2:2, 1	262	II	<i>P. savastanoi</i> (77-100)	R5 (81-90)
2:2, 2	6	V	<i>E. herbicola</i> (88-94)	R2 (82-95)
2:2, 3	9	VIII	<i>B. megaterium</i> (79-98)	R1 (76-90)
2:2, 4	4	IX	<i>M. luteus</i> (91-97)	R12 (78-88)
2:3, 1	4	II	<i>P. savastanoi</i> (88-96)	R5 (77-94)
2:3, 2	9	IV	<i>X. campestris</i> group (78-95)	R6 (76-89)
2:3, 3	7	V	<i>E. herbicola</i> (96-98)	R2 (83-91)
2:3, 4	6	X	<i>S. marcescens</i> (88-97)	R15 (79-86)
2:3, 5	11	VIII	<i>B. megaterium</i> (82-99)	R1 (78-91)
2:3, 6	4	XI	<i>Le. dextranicum</i> (88-100)	R11 (78-84)
2:3, 7	6	XII	<i>La. plantarum</i> (84-96)	R4 (78-89)
2:3, 8	3	XIII	<i>Ar. globiformis</i> (97-99)	R8 (78-85)
2:3, 9	15	IX	<i>M. luteus</i> (84-100)	R12 (75-91)
2:4, 1	62	II	<i>P. savastanoi</i> (78-100)	R5 (80-92)
2:4, 2	11	XIV	<i>Ac. aceti</i> (84-100)	R7 (80-93)
2:4, 3	28	V	<i>E. herbicola</i> (78-100)	R2 (77-94)
2:4, 4	4	XI	<i>Le. dextranicum</i> (86-96)	R11 (77-89)
2:4, 5	6	XII	<i>La. plantarum</i> (85-98)	R4 (77-92)
2:4, 6	11	XIII	<i>Ar. globiformis</i> (89-97)	R8 (76-90)
2:4, 7	3	IX	<i>M. luteus</i> (92-98)	R12 (79-86)
3:1, 1	11	II	<i>P. savastanoi</i> (80-97)	R5 (80-94)
3:1, 2	7	III	<i>P. fluorescens</i> (95-99)	R14 (79-87)
3:1, 3	4	IV	<i>X. campestris</i> group (78-92)	R6 (78-88)
3:1, 4	9	V	<i>E. herbicola</i> (86-100)	R2 (80-97)
3:1, 5	9	VI	<i>K. pneumoniae</i> (86-95)	R10 (78-84)

Table 4 (cont.)

Set and cluster no.*	No. of isolates in cluster	Aggregate cluster no.†	Identification of type isolate in aggregate cluster and its %S range with other isolates in the cluster	Reference culture‡ and %S range with isolates in the cluster
3:1, 6	2	VII	<i>B. subtilis</i> (100)	R9 (80)
3:1, 7	7	VIII	<i>B. megaterium</i> (83–100)	R1 (78–89)
3:1, 8	2	IX	<i>M. luteus</i> (100)	R12 (78)
3:2, 1	409	II	<i>P. savastanoi</i> (76–100)	R5 (76–89)
3:2, 2	4	V	<i>E. herbicola</i> (92–99)	R2 (88–96)
3:2, 3	5	VIII	<i>B. megaterium</i> (84–97)	R1 (75–88)
3:3, 1	2	II	<i>P. savastanoi</i> (100)	R5 (90)
3:3, 2	11	IV	<i>X. campestris</i> group (80–91)	R6 (75–90)
3:3, 3	8	V	<i>E. herbicola</i> (89–95)	R2 (80–97)
3:3, 4	10	X	<i>S. marcescens</i> (85–100)	R15 (78–89)
3:3, 5	9	VIII	<i>B. megaterium</i> (82–96)	R1 (79–88)
3:3, 6	5	XI	<i>Le. dextranicum</i> (87–96)	R11 (80–88)
3:3, 7	4	XII	<i>La. plantarum</i> (91–95)	R4 (80–92)
3:3, 8	4	XIII	<i>Ar. globiformis</i> (90–96)	R8 (81–88)
3:3, 9	16	IX	<i>M. luteus</i> (85–99)	R12 (77–93)
3:4, 1	46	II	<i>P. savastanoi</i> (78–100)	R5 (80–95)
3:4, 2	5	IV	<i>X. campestris</i> group (80–97)	R6 (75–84)
3:4, 3	8	XIV	<i>Ac. acetii</i> (85–97)	R7 (81–92)
3:4, 4	33	V	<i>E. herbicola</i> (77–99)	R2 (75–97)
3:4, 5	2	VIII	<i>B. megaterium</i> (99)	R1 (83–84)
3:4, 6	3	XI	<i>Le. dextranicum</i> (92–99)	R11 (86–92)
3:4, 7	2	XII	<i>La. plantarum</i> (94)	R4 (77–82)
3:4, 8	7	XIII	<i>Ar. globiformis</i> (88–98)	R8 (80–91)
3:4, 9	4	IX	<i>M. luteus</i> (88–97)	R12 (81–91)

* Sets 1:1 to 1:4, 2:1 to 2:4 and 3:1 to 3:4 contained the isolates obtained in January, April, July and October during the first, second and third years of study, respectively.

† Each aggregate cluster included the isolates from clusters that generated hypothetical median organisms (Oliver & Colwell, 1974) linked at $\geq 70\%$ S.

‡ See Table 2.

identified to the genus level or below according to Buchanan & Gibbons (1974), with the exception of HR-positive pseudomonads that were identified to the species level according to Sands *et al.* (1970).

In the sequence described above the first step determined the existence and size of clusters of phenetically highly related isolates within each set, steps (ii) and (iii) determined the quantitative variations of different phenetic groups in time and step (iv) identified the isolates representative of the defined phenetic groups.

In control experiments performed at the end of the work described above, the reference cultures R7 to R15 were characterized as before. From these, and the data for R1, R2, R4, R5 and R6, the S_j value was calculated for each of the pair comparisons between the isolates in each aggregate cluster and the reference culture that represented the taxon to which the type isolate of the aggregate cluster had been assigned. When all reference cultures were added to each of the seasonal sets and the sequence (i) to (iv) described above was repeated, the distribution of the isolates among the various clusters remained unchanged. The similarity matrix for the isolates in cluster 1:1 and the reference cultures R1 to R6 is shown in Fig. 1.

RESULTS

The distribution of the isolates to clusters within each set is shown in columns 1 and 2 of Table 4. Over 97% of the isolates, 1744 out of 1789, were recovered in clusters defined at or above the 70% S level. In fact, mean intracluster and intercluster similarity values were invariably greater than 85% and smaller than 60%, respectively. The three sets obtained in April were the most homogeneous. Approximately 96% of the isolates in these sets fell in one cluster, the remainder being distributed in two or three additional clusters. In contrast, the January and July sets were, with one exception, the most heterogeneous with no more than 23% of the isolates contained in any one of the seven to nine clusters defined within each set. The exception was set 1:1 in which approximately 33% of the isolates were recovered in a single cluster. The October sets also contained between seven and nine clusters, and the

largest two contained, on average, approximately 70% of the isolates. The proportion of isolates that were not clustered was highest in January (15.7% in 1973, 12.5% in 1974 and 13.6% in 1975) and intermediate in July (3.6, 4.4 and 5.5%) and October (1.0, 3.8 and 1.8%). In the April samples all of the isolates were recovered in defined clusters.

The isolates in the defined clusters were arranged into 14 aggregate clusters as shown in column 3 of Table 4. Most of the aggregate clusters contained between 19 and 72 isolates but a minimum of 2 and a maximum of 1214 isolates were recorded in aggregate clusters I and II, respectively. Mean similarity values > 82% and < 60% were invariably recorded within and between aggregate clusters, respectively.

The identification of the type isolate of each aggregate cluster and the range of S values between the isolates in each cluster and the appropriate reference culture are shown in columns 4 and 5 of Table 4. Using these data (see Discussion) isolates in aggregate cluster IV were equated to the *Xanthomonas campestris* group and those in aggregate clusters I to III and V to XIV were identified as *Pseudomonas delafieldii*, *P. savastanoi*, *P. fluorescens*, *Erwinia herbicola*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *B. megaterium*, *Micrococcus luteus*, *Serratia marcescens*, *Leuconostoc dextranicum*, *Lactobacillus plantarum*, *Arthrobacter globiformis* and *Acetobacter aceti*, respectively. These data combined with those in columns 1 to 3 of Table 4 show that the distribution of the named bacteria differed considerably among the sets obtained in the different seasons of a year, but not among those obtained during the same season in different years.

Rearrangement of the data from Table 4 shows that the average number of named isolates regularly encountered at different times of the year were: *B. megaterium* (6), *E. herbicola* (10), *K. pneumoniae* (8), *P. fluorescens* (6), *P. savastanoi* (16) and the *X. campestris* group (6) in January; *B. megaterium* (7), *E. herbicola* (5) and *P. savastanoi* (334) in April; *Ar. globiformis* (4), *B. megaterium* (9), *E. herbicola* (7), *La. plantarum* (4), *Le. dextranicum* (4), *M. luteus* (15), *P. savastanoi* (3), *S. marcescens* (8) and the *X. campestris* group (9) in July; and *Ac. aceti* (7), *Ar. globiformis* (9), *E. herbicola* (29), *La. plantarum* (4), *M. luteus* (3) and *P. savastanoi* (52) in October. Other isolates recovered in two of the three years were: *B. subtilis* (3) and *M. luteus* (3) in January; *M. luteus* (4) in April; and *B. megaterium* (2), *Le. dextranicum* (4) and the *X. campestris* group (7) in October. Gram-positive bacteria were the most common (52.1 to 57.4%) isolates obtained in July, fell to a minimum (1.2 to 4.6%) in April, and remained approximately constant in January (12.9 to 18.6%) and October (16.1 to 18.5%). Pigmented isolates, comprising aggregate clusters II, III, IV, IX, X, XIII and part of aggregate cluster V, formed 44.1 to 51.4% of the total in January, 57.4 to 66.1% in July, 62.5 to 69.2% in October, and 95.4 to 98.1% in April.

The seasonal fluctuations in the populations of most of the named bacteria fell into recognizable patterns (Fig. 2). Thus, *P. savastanoi* and *E. herbicola* were found in all of the samples, but the former increased 14- to 37-fold from January to April, decreased to between 0.7×10^3 and 1.3×10^3 viable bacteria cm^{-2} in July and, in October, increased again to levels that were from two to four times as high as those in January. In contrast, the numbers of *E. herbicola* remained approximately constant in January, April and July and increased 2.33- to 8.25-fold in October. Bacteria identified in more than half of the sets included the *X. campestris* group which always fell below the detection level in April, *B. megaterium* that invariably declined in October and *M. luteus*, populations of which were above average in July. *Arthrobacter globiformis* and *La. plantarum* were only regularly found in July and October, the former differing from the latter by showing higher populations in October than in July. A pattern of seasonal fluctuation similar to that of *La. plantarum* was also exhibited by *Le. dextranicum*, although the latter fell below the threshold level in October 1973. Most of the remaining bacteria were consistently encountered at yearly intervals: *K. pneumoniae* and *P. fluorescens* in January, *S. marcescens* in July and *Ac. aceti* in October. *Bacillus subtilis* was only detected in January 1973 and 1975, and *P. delafieldii* in January 1973.

Fig. 2. Seasonal fluctuation in the populations of taxa found on the phylloplane of the olive.

DISCUSSION

Current views on the types of bacteria to be expected on the aerial parts of plants (Billing, 1976) make it reasonable to assume that the selection procedure adopted for the isolation of the aerobic, heterotrophic, mesophilic strains should not have been biased against any major component of the bacterial community. Determination of the bacterial populations using 10^{-2} instead of 10^{-3} dilutions in January, July and October would have lowered the threshold for the detection of different bacterial types but would have resulted in an almost unmanageable number of isolates and, more importantly, the comparison between the sets obtained in April and at other times of the year would have been unbalanced.

Numerical taxonomic methods afford an effective way of monitoring variation in populations of phylloplane bacteria (Goodfellow *et al.*, 1976; Austin *et al.*, 1978) but they present the user with a choice of alternatives which may affect their outcome. In the present study, the S_j coefficient, which excludes negative matches, was preferred to the simple matching coefficient (S_{SM} ; Sokal & Michener, 1958), which includes both positive and negative matches, to minimize differences arising from inapplicable tests (Sneath, 1972). As the S_j coefficient emphasizes differences between isolates (Austin & Colwell, 1977), single linkage instead of average linkage cluster analysis was used throughout the study. The analysis was intended to find preferentially (Sneath, 1972) elongated aggregate clusters expected to include the isolates of major taxa obtained at different times of the year, but control experiments showed that it defined virtually the same clusters as the unweighted pair group method with arithmetic averages (Sneath, 1972). The analysis method chosen was easily programmed and quickly carried out on the computer. Test error and reproducibility were not monitored but statistical analysis of the 12 replicate sets of test data available for the reference cultures R1 to R6 indicated that there was an average probability of erroneous test results (p ; Sneath & Johnson, 1972) of 1.435%. This, combined with the recovery of isolates in well-defined homogeneous clusters, suggests that test error remained within acceptable limits.

The numerical phenetic method used provided a continuous way of monitoring the efficiency of the approach for identifying the phylloplane bacteria and measuring their seasonal variation. Thus, mean similarity values of $> 85\%$ and $< 60\%$ within and between clusters, respectively, indicated that the clustering procedure was successful in recovering well-isolated 'species level' groupings (Colwell & Liston, 1961; Liston *et al.*, 1963; Colwell & Mandel, 1964) from the seasonal sets. In addition, the formation of aggregate clusters showing mean intracluster and intercluster similarity values of the same order of magnitude suggested that the degree of inter-relatedness among the isolates in the same aggregate cluster, and the degree of their separation from other isolates, were similar to those expected within and between 'species level' groupings, respectively. The finding that all of the isolates in an aggregate cluster formed a well-defined phenon with the reference culture representing the type isolate justified naming the group after the reference culture (Skerman, 1967). A limited number of reference cultures was included in this study but the successful identification of all the phenons suggested that the criterion for their choice was effective; however, the same criterion may be of limited value under different circumstances (Austin *et al.*, 1978).

It is difficult to compare the results of the present study with earlier work on phylloplane bacteria because of differences both in methodology and the type of plant studied. However, the finding that only a few taxa were present on olive leaves at any given time of sampling was in good agreement with previous studies (Dickinson *et al.*, 1975; Goodfellow *et al.*, 1976; Austin *et al.*, 1978). The frequency of pigmented isolates on leaves was also in line with earlier reports (Voznyakovskaya & Khudyakov, 1960; Stout, 1960; Jensen, 1971; Klincäre *et al.*, 1971; Dickinson *et al.*, 1975; Austin *et al.*, 1978). Arginine dihydrolase-negative fluorescent plant pathogenic pseudomonads comprise several well-recognized epiphytes (Leben, 1965, 1974; Schuster & Coyne, 1974) and such strains, identified as *P. savastanoi*, accounted for approximately two-thirds of all the isolates obtained in the present

survey. Despite differences in methodology, the recovery and incidence of *P. savastanoi* strains from olive leaves at different times of the year did not differ appreciably from those recorded in earlier studies (Ercolani, 1971), suggesting that the recurrent concomitance between high density of the pathogen in the phylloplane and cambial activity of the host may be responsible for the observed increase in the infection pressure of olive knot disease in the spring and autumn.

The second most frequently encountered taxon on olive leaves was *E. herbicola* which also includes plant surfaces among its natural habitats (Murata & Starr, 1974; Billing, 1976). Less than a quarter of the olive phylloplane isolates were distributed among the following taxa with decreasing frequency: *B. megaterium*, *M. luteus*, the *X. campestris* group, *Ar. globiformis*, *La. plantarum*, *K. pneumoniae*, *S. marcescens*, *Ac. aceti*, *Le. dextranicum*, *P. fluorescens*, *B. subtilis* and *P. delafieldii*. Several of these taxa have been reported in previous surveys, i.e. *P. fluorescens* (Voznyakovskaya & Khudyakov, 1960; Goodfellow *et al.*, 1976; Austin *et al.*, 1978), the *X. campestris* group (Leben, 1963; Goto, 1972; Austin *et al.*, 1978), *La. plantarum* (Pederson, 1936; Voznyakovskaya & Khudyakov, 1960), *B. megaterium* (Stout, 1960; Voznyakovskaya & Khudyakov, 1960), *B. subtilis* (Stout, 1960) and *M. luteus* (Voznyakovskaya & Khudyakov, 1960). *Klebsiella pneumoniae* has been reported from habitats as different as forest trees and farm produce (Duncan & Razzell, 1972; Brown & Seidler, 1973) but its true epiphytic nature has not been established until now. Although there appear to be no previous reports of *Le. dextranicum* from the phylloplane its presence on olive leaves was not surprising in view of the recognized ability of several lactic acid bacteria to colonize plant surfaces (Mundt *et al.*, 1962; Stirling & Whittenbury, 1963). The discontinuous occurrence of *S. marcescens* and *Ac. aceti*, which were only isolated from olive leaves in July and October, respectively, may explain their omission in previous surveys of the phylloplane flora.

Several factors, such as leaf age and nutrition and the source of colonizing bacteria, may influence the density of bacterial populations on olive leaves and thereby affect their seasonal fluctuation. At present it is not known whether the bacterial populations on olive leaves occur in the olive environment at large or whether they fluctuated in alternative niches at the same rate as on the phylloplane. It is also not yet possible to draw precise correlations between environmental parameters and the effectiveness of a given bacterium in colonizing the phylloplane, but the consistency in the pattern of seasonal fluctuation in bacterial populations over the three years strongly suggests that the dynamics of different bacterial populations present on the phylloplane at any given time are governed by regularly recurrent events in their habitat. The fact that isolates of *P. savastanoi* obtained at different times of the year differed in several cultural, physiological and biochemical characters also suggests that the relative efficiency of the bacteria in utilizing the leaf surface as a substrate for growth and survival may be an important factor in this respect. Material originating outside the leaf may improve the nutrient value of the phylloplane for some micro-organisms (Fokkema, 1971; Yoder & Whalen, 1975; Clark & Lorbeer, 1976), but a significant role for pollen on the phylloplane of the olive does not seem likely as control experiments resulted in a lower recovery of bacteria from olive leaves in May, when the olive and most grasses were flowering in the area, than in April. A similar conclusion was reached by Dickinson *et al.* (1975) in their study of phylloplane bacteria from *Lolium perenne*.

The relevance of most phylloplane bacteria in the biology of the olive has still to be determined. *Klebsiella pneumoniae* is able to fix nitrogen but it is unlikely that the environmental conditions for this (Pengra & Wilson, 1958; St John *et al.*, 1974) exist on the phylloplane of the olive. Further work is also required to determine whether the systematic occurrence of *Ac. aceti*, *La. plantarum* and *Le. dextranicum* on the phylloplane of the olive in October is related to their presence on the surface of ripening olive fruit at harvesting, a fact that may have significant effects on the fermentation of such fruit in the pickling industry (Cruess, 1930; Vaughn, 1954). Finally, since *P. savastanoi* does not readily infect olive

leaves, future studies should clarify the lack of correlation between the ability to build high populations on olive leaves and what is considered a prerequisite of high virulence, i.e. multiplication in the host tissues from small inocula (Ercolani & Crosse, 1966; Ercolani, 1973).

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