AN ABSTRACT OF THE THESIS OF

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Title: PATHOGENIC AND GEL ELECTROPHORETIC COMPARISON OF PSEUDOMONADS FROM FRUIT TREES

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Sixty-two isolates of Pseudomonas, principally from fruit trees, were compared on the basis of LOPAT characteristics, pathogenicity, and protein band pattern produced by gel electrophoresis of soluble proteins. All but one of the oxidase negative isolates fell into LOPAT group Ia. The oxidase positive isolates were placed into groups IVa, Va, and Vb, with the majority being in group Va.

Pathogenicity tests utilized cherry buds in the field and excised green fruits of cherry, prune, and pear in the laboratory. None of the oxidase positive isolates were pathogenic. Of the oxidase negative isolates, only Pseudomonas syringae and P. morsprunorum killed intact buds. Other pathogenic nomenspecies as well as some isolates of P. syringae were avirulent under these conditions. All of the P. syringae isolates were pathogenic on the three types of fruits. P. morsprunorum isolates were mildly pathogenic on cherry and prune but not on pear fruits. P. glycinea and P. phaseolicola also caused slight reactions on cherry fruits but not on the others.

Electrophoretic data confirm the close relationship of the oxidase

negative plant pathogens, as well as their distinction from the saprophytic species. Twenty-nine isolates of P. syringae had very similar but not identical protein band patterns. Band patterns of P. syringae, P. morsprunorum, P. lachrymans, P. glycinea, and P. phaseolicola were all similar but distinguishable from each other. The data support the placing of these oxidase negative plant pathogens in a single taxospecies but recognizing them as separate nomenspecies. The oxidase positive isolates were divided into several tentative groups on the basis of band pattern.

PATHOGENIC AND GEL ELECTROPHORETIC COMPARISON OF PSEUDOMONADS FROM FRUIT TREES

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PATHOGENIC AND GEL ELECTROPHORETIC COMPARISON OF PSEUDOMONADS FROM FRUIT TREES

INTRODUCTION

New techniques and additional information have led to numerous changes in the taxonomy of phytopathogenic bacteria over the years. While generic concepts are becoming more stable, species are still poorly defined. Numerical taxonomy (Stolp et al., 1965), DNA base composition (DeLay et al., 1966, Jackson and Sands, 1970), as well as biochemical tests, have been used to group related organisms that might have formerly merited separate specific rank into fewer species. Tests now commonly employed generally cannot distinguish between species erected solely on the basis of host plant. In some cases plant pathogens cannot be distinguished from saprophytes. Several recent reviews have dealt with this problem (Marmur et al., 1965; Mandel, 1969).

Among the plant pathogens the Pseudomonas syringae-P. morsprunorum complex has been studied extensively over the years. Earlier work has been reviewed by Cameron (1962). Relationships within the species, between P. syringae and other plant pathogens, as well as the relationships between the pathogenic and saprophytic species are areas of current interest.

A number of different types of studies have all indicated the complexity of the P. syringae group. Phage typing (Billing, 1963; Garrett et al., 1966; Lazar and Crosse, 1969), serology (Perlasca, 1960; Lovrekovich et al., 1963; Burki, 1968; Guthrie, 1969; Otta and English, 1971), and pathogenicity tests (Burki, 1968; Crosse, 1966) have all been used when comparing isolates of P. syringae and P. morsprunorum as well as for dividing these species into recognizable groups at the intra-

specific level. Otta and English (1971) for example, recognized at least 10 serological groups in the species complex. Nutritional and biochemical tests have also confirmed the heterogeneity of the species (Shackleton, 1968; Baker, 1966; Huber et al., 1970).

While P. syringae and P. morsprunorum themselves constitute a complex group of organisms, it is evident that they are part of a closely related group of plant pathogenic nomenspecies. On the basis of nutritional and biochemical tests, Lelliott et al. (1966) and Misaghi and Grogan (1969) placed almost all of the oxidase negative plant pathogenic species they tested into a single group. Oxidase positive saprophytes from plant material, including isolates of P. fluorescens and P. aeruginosa, were also shown to form a closely related group that was clearly distinct from the plant pathogens. Jackson and Sands (1970) showed that the plant pathogens had similar DNA base compositions, which also indicates a close relationship among these organisms.

In addition to the methods mentioned above, comparative band patterns produced by gel electrophoresis of soluble proteins have recently been used as a taxonomic guide. Several studies have been made on higher organisms but the method seems to be particularly applicable to microorganisms. Fungi have been studied by a number of workers, as have several genera of bacteria. The latter include Xanthomonas (Gill and Khare, 1968; El-Sharkawy and Huisingh, 1968), Agrobacterium (Huisingh and Durbin, 1967), Corynebacterium (Robinson, 1966), Erwinia (Smith and Powell, 1968), Streptococcus (Lund, 1965), and Streptomyces (Gottlieb and Hepden, 1966). Hoitink et al. (1968) also used electrophoresis when comparing two species of Pseudomonas.

The purpose of this study was to compare fluorescent pseudomonads from fruit trees in the Willamette Valley with other pathogens and saprophytes from around the world on the basis of pathogenic characteristics and protein patterns produced by gel electrophoresis.

MATERIALS AND METHODS

Cultures

Cultures of Pseudomonas were isolated from trees at the Plant Pathology farm, Corvallis, Oregon, or from diseased trees in other areas of the Willamette Valley by the author and others (Cameron, 1970). Additional isolates were supplied by other laboratories. The isolates of P. fluorescens and P. aeruginosa were included for comparative purposes. Table 1 lists the isolates with their source, original host and geographic location. Each isolate was purified by streaking on a plate of King's medium B (King et al., 1954). Single fluroescent colonies from plates having homogeneous appearing colonies were selected to prepare stock cultures. These stock cultures were stored in sterile distilled water at room temperature according to the method of DeVay and Schnathorst (1963).

LOPAT test

Since growth characteristics indicated the isolates being studied formed a heterogeneous grouping, each isolate was subjected to the "LOPAT" test of Lelliott et al. (1966), and grouped accordingly. This test constituted a basis for comparison to be used in the electrophoretic studies described below. Characters used in the test are levan production, presence of oxidase, ability to rot potato slices, presence of arginine dihydrolase, and a hypersensitive reaction to tobacco leaves.

Table 1. Host, isolate designation, and source of *Pseudomonas* isolates.

Host	Isolates	Obtained From
	Pseudomonas syringae	
Cherry	940-R[b], 919-S[b]	California
	Ps-144[b]	Hungary
	43-1, 43-2, SC-1, SC-2, SC-4	Oregon
	GW-4, GW-8, GW-9, GW-13	"
Prune	GS-3S[b], 7f-S1[b], 857-S2[b]	California
	GG-1, GG-2, Fr. prune	Oregon
Pear	P-2, P-3	Oregon
	B-301[b], E-3[b]	England
	S-30t[b]	California
Peach	B-3[d], B-3AS[b]	California
Almond	912-S2[b]	"
Lilac	LM-1	Oregon
Bean	HP-C[b]	Wisconsin
Unknown	Syr D[c]	Missouri
	Pseudomonas sp.	
Cherry	20-1, H-2C, 47-48-1, 86-27	Oregon
	8-1-2, 8-5,-1, 8-19-3, 8-19-4	"
	8-40-8, 86-39, 48a, 14, 15, 19	"
	SB-5, SB-6, H-4	"
	S-3, S-4, S-6, S-7	Sweden
	Pseudomonas morsprunorum	
Plum	M.P.[c]	England
	B-299[b]	"
	Pseudomonas phaseolicola	
Bean	9-B[e]	New York
	21-B[e]	"
	SV-2[e]	Oregon

Table 1. (Continued)

Host	Isolates	Obtained From
Cucumber	*P. lachrymans*	Oregon
Soybean	*P. glycinea*[c]	Missouri
	P. fluorescens	Oregon
	P. aeruginosa	"
Citrus	94R[b]	California

b Obtained from Jack Otta, University of California, Davis.

c Obtained from R.N. Goodman, University of Missouri.

d Obtained from J.E. DeVay, University of California, Davis.

e Obtained from E.K. Vaughan, Oregon State University, Corvallis.

Cultures not designated were isolated by the authors or obtained from others at Oregon State University, Corvallis.

Levan production.

Isolates were streaked onto plates of Difco nutrient agar containing 5% sucrose. The development of large, white, domed mucoid colonies indicated the production of levan.

Presence of oxidase.

A loopful of bacteria from a 24 hour culture grown on medium B was smeared on an oxidase differentiation disk (Difco, 1633-35). The development of a purple to black color in 10-30 seconds was recorded as positive. Since the liquid reagent of Kovacs(1956) is normally used in this test, a comparison was made between this reagent (N, N'-dimethyl-p-phenylenediamine) and the Difco disks. Results proved to be identical and since the disks were more convenient, they were used throughout this study.

Potato soft rot.

Tubers were washed, alcohol flamed and peeled with a flamed peeler. Slices 8-10 mm thick were placed in petri dishes, with two slices per dish. These were immediately flooded with sterile distilled water so that the water was about half way up on the slice. The slices were heavily inoculated with growth from a 24 hour slant culture by placing the bacteria in a notch made in the center of each slice. Slices were tested for soft rot after two and three days by stabbing with an inoculating needle. Uninoculated slices served as controls.

Presence of arginine dihydrolase.

8 x 75 mm test tubes were filled to a depth of 18 mm with Thornley's (1960) arginine medium 2A. Stab inoculations were made in each tube, then the surface of the medium was covered with sterile melted petroleum jelly to provide anaerobic conditions. The tubes were observed after 24 and 48 hours incubation at room temperature. The development of a red color in the medium, indicating the conversion of arginine to alkali, was considered positive for the presence of the enzyme.

Tobacco hypersensitivity test.

The tobacco variety Samsun was used. The plants were grown to the early flowering stage and the lowest nine healthy leaves on each plant were used. Nutrient dextrose broth cultures 12-18 hours old adjusted to a density of about 10^8 cells per ml were used as inoculum. Half leaves were used for each isolate and each half leaf was inoculated to give at least three infiltrated areas two cm or larger in diameter. Klement's (1963) method of inoculation was used.

Pathogenicity tests

Information on pathogenicity and relative virulence of the pathogenic isolates was obtained through field inoculations of cherry trees and laboratory inoculations of green fruits.

Field tests.

Field studies were conducted at the Oregon State University Plant

Pathology farm east of Corvallis using four- to five-year old sweet cherry trees of the variety Napoleon. Inoculations were made during the first week of December, 1969. The first 10-12 buds behind the terminal bud of each shoot were nicked with a razor blade, then a drop of a 12-18 hr nutrient glucose broth suspension containing about 10^8 cells per ml was placed on the wound. A separate shoot was used for each isolate and each isolate was inoculated into three different trees, making a total of 35 buds inoculated with each isolate. Controls consisted of branches treated the same except that sterile nutrient glucose broth was placed on the wound. This rather severe method of inoculation was used in an effort to identify supposedly less virulent strains of Pseudomonas syringae causing "Dead Bud" type symptoms but not canker (Cameron, 1960). Observations to determine pathogenicity and virulence were made the following spring at the time of bud break.

Laboratory tests.

Green fruits of Napoleon cherry, Early Milton prune and Bartlett pear were used. The fruits were washed in tap water and air dried. Inoculum consisted of 24 hour cultures grown on King's medium B. Inoculations were made by picking a small amount of bacteria from the plate on the point of a previously sterilized needle and puncturing the surface of the fruit. Fruits pierced with a sterile needle served as controls. Each pear fruit was inoculated three times, each prune fruit twice, and each cherry fruit once. Five fruits were inoculated with each isolate. The fruits were set on petri dish bottoms and placed in small plastic boxes with a wet paper towel in the bottom to maintain

high humidity. Incubation was at 15° C for one week. Virulence ratings were based on an arbitrary scale having a range of zero to four in cherries and prunes, and zero to three in pears. A rating of zero indicated no visible symptoms, while a rating of four indicated large, sunken, necrotic lesions. The other ratings indicated various degrees of necrosis between the two extremes. To confirm the fact that necrosis was caused by pathogenic pseudomonads, reisolations were made from about one-third of the inoculated fruits by surface sterilizing the fruits in 10% chlorox, then plating out bits of tissue from the margin of lesions onto plates of medium B. Fluorescent colonies that grew were then tested for the presence of oxidase as described previously.

Protein electrophoresis

Cultures for electrophoresis studies were grown for three days at 25° C in one-liter flasks containing 400 ml of nutrient glucose broth (3 g beef extract, 10 g peptone, 10 g glucose per liter). At the time of harvesting the cultures were checked for purity by streaking a sample from each flask on a plate of medium B agar. Cells were harvested by centrifuging for 10 minutes at 5800 x g, washing in 50 volumes of 0.05 M Tris [tris (hydroxymethyl) aminomethane] - HCl buffer at pH 7.2, and centrifuging again. Preliminary experiments were conducted comparing Tris, HEPES (N-2-hydroxy ethyl piperazine-N'-s-ethane sulfonic acid) and phosphate buffers over a range of pH's to determine the optimal conditions.

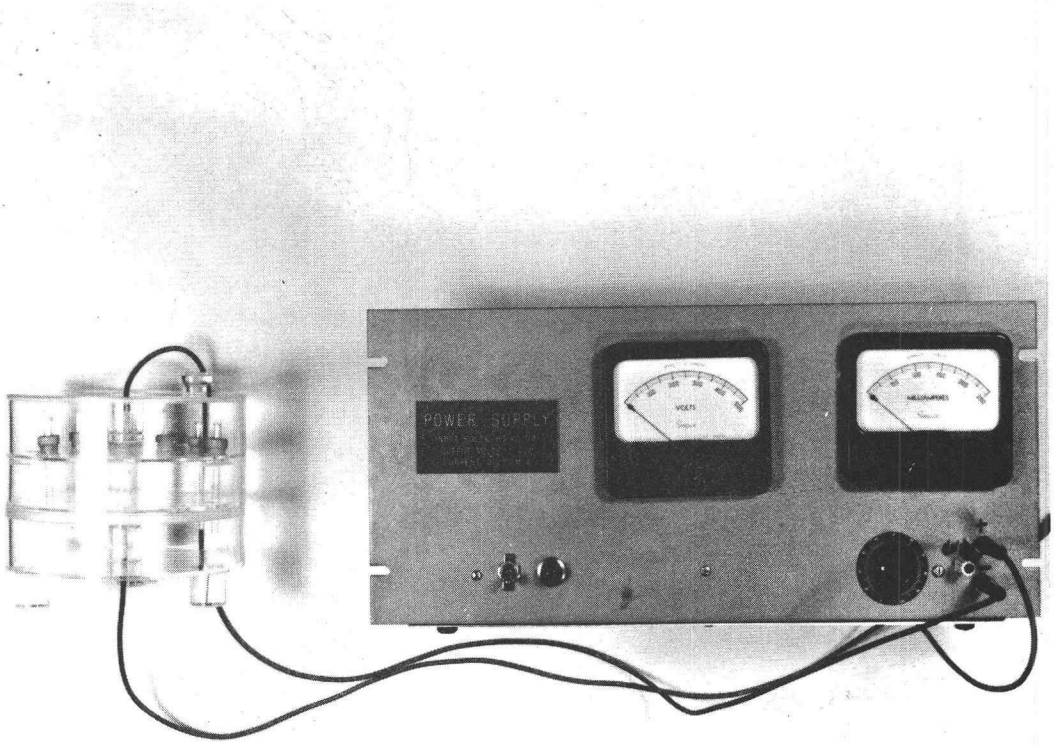
Soluble proteins were extracted by suspending the cells in equal volumes of Tris buffer and sonicating with an MSE ultrasonic disintegrator

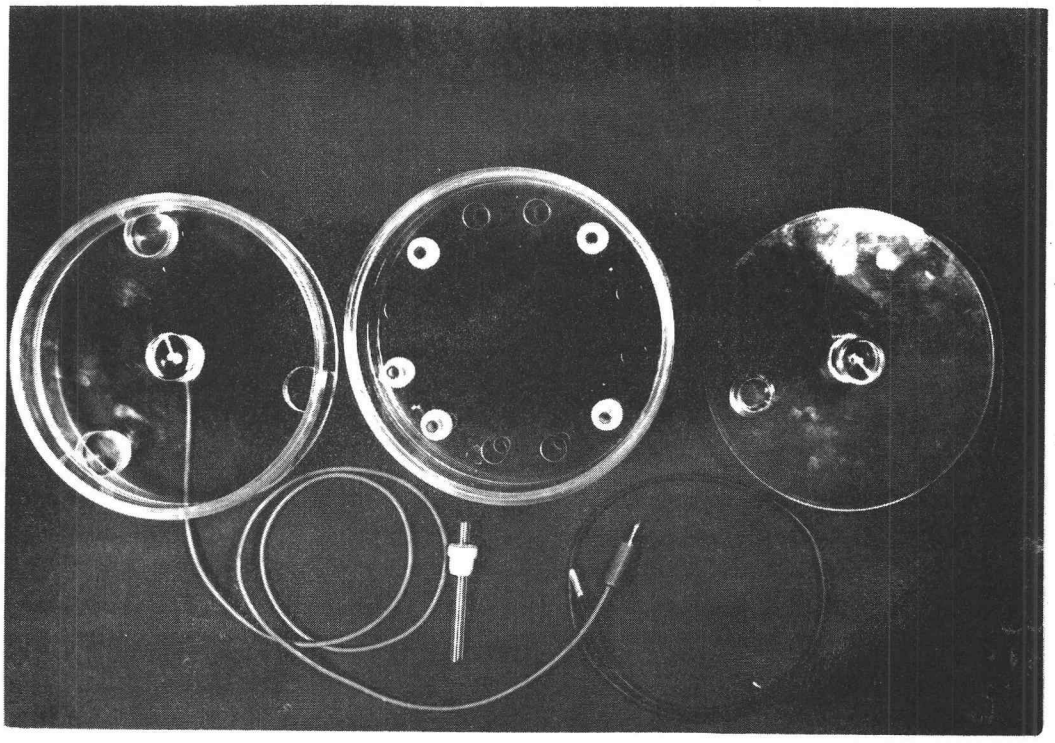
for 3.5 minutes. To prevent heating during sonication the suspension was surrounded by dry ice and sonicated for 20 second intervals alternating with 5-8 second pauses for cooling. The broken cell suspension was centrifuged at 4° C for 60 minutes at 100,300 x g in a Spinco model L ultracentrifuge. The supernatent containing the proteins was divided into 0.5 ml aliquots and frozen at -20° C until needed.

Acrylamide gels were prepared according to the method of Davis (1964), using tubes 0.5 x 8.0 cm. Both a spacer gel and a sample gel were used. Each tube contained about 500 µg protein as determined by the method of Lowry et al. (1951). Bovine serum was used as the standard. The reservoir buffer in the electrophoretic chamber was diluted 1:1 rather than 1:10 as in Davis' (1964) standard method, since at the higher dilution excess voltage was required, which led to an increase in temperature. The electrophoretic apparatus used was built by the Oregon State University Physics shop. It is shown in Figures 1 and 2.

Electrophoresis was conducted at 2.0 milliamps per tube for five minutes, then at 4.6 milliamps per tube until the front, as indicated by the bromphenol blue marker dye, had moved about four cm through the running gel. Gels were removed from the tubes by rimming the gels with a jet of water shot through a hypodermic needle. They were then fixed for 40 minutes in 12.5% trichloroacetic acid, and stained for 25 minutes with Coomassie Brilliant Blue R 250 according to the method of Chrambach et al. (1967). Destaining was carried out for two days in 10% trichloroacetic acid, after which the gels were transferred to 7% acetic acid for permanent storage. Densitometer tracings of the gels were made with a Schoeffel model SD-3000 spectrodensitometer set at a

Figures 1-2. Electrophoretic apparatus. 1. Complete unit with electrophoretic chamber on the left and power supply on the right. 2. Disassembled chamber. Left, lower buffer reservoir with anode. Center, upper buffer reservoir with several tubes in position. Right, lid with cathode. Lower center, gel tube with grommet made from a Vacutainer stopper.


POWER SUPPLY
VOLTS
MILLIAMPERES



wavelength of 650 nm.

Comparisons were made between duplicate gels of the same extract, portions of the same extract that were frozen for different lengths of time, and different extractions from the same isolate. In addition, band positions and intensities of 1:1 protein mixtures of paired isolates were compared with those of each individual isolate (Johnson, et al., 1967). In this way homologous bands (i.e., bands with the same migration velocity) could be established, even though there were small, uncontrollable differences in the actual migration velocities of the protein bands in some of the gels. Once two or more widely separated pairs of bands per pair of gels was established, the densitometer tracings were adjusted photographically to show the homologous bands at equivalent points. A comparison of bands from different isolates could then be made.

RESULTS

LOPAT Test

Based on the results of the LOPAT tests, the isolates could be divided among five of the groups of Lelliott et al. (1966). 36 of the isolates were placed in group Ia, one in group I?, two in group IVa, 15 in group Va, and eight in Vb (Table 2). All but one of the named isolates of Pseudomonas syringae, P. morsprunorum, P. phaseolicola, P. glycinea, and P. lachrymans, as well as 15 isolates obtained locally, belonged to group Ia. One isolate, 94-R, isolated from Citrus sp. and received as P. syringae, did not cause a hypersensitive reaction in tobacco. Accordingly, it was placed in the questionable group I of Lellott et al. (1966). Since it also proved to be non-pathogenic on cherry, prune, and pear it was excluded from the P. syringae group. Otta, who supplied this isolate also later confirmed by serology that it was not P. syringae (Otta, personal communication).

The two isolates belonging to group IVa were both isolated from cherry trees in the Willamette Valley. Isolate 20-1, from cherry wood, showed weakly positive oxidase and arginine dyhydrolase tests, so its grouping may be questionable. Repeated tests gave the same results, however. Isolate H-2C was from cherry buds showing dead-bud symptoms, but it proved to be non-pathogenic in all of the pathogenicity tests conducted.

The majority of the oxidase positive isolates from the Plant Pathology farm, as well as the named isolates P. aeruginosa and P. fluorescens, belonged to group Va. Group Vb included isolates S-3,

Table 2. LOPAT reactions of *Pseudomonas* isolates

Isolate	levan production	oxidase	potato rot	arginine	tobacco	group
43-1, 43-2, B-3 B-3AS, SC-1, SC-2, SC-4 GG-1, GG-2, B-301, GS-3S, GW-4, GW-8, GW-9, GW-13, E-3, LM-1, P-2, P-3, S-30t Syr D, 912-S2, 919-S, 940-R, 7f-S1, 857-S2, HPC, Ps 144, Fr pr, *P. morsprunorum*, *P. glycinea*, *P. phaseolicola*, *P. lachrymans*	+	-	-	-	+	Ia
94-R	+	-	-	-	-	I?
20-1, H2-C	+	+	+	+	-	IVa
8-1-2, 8-5-1, 8-19-3 8-19-4, 8-40-8, 14, 38-20-1, 47-48-1, 48a, 86-27, 86-39, 86-49, SB-6, *P. fluorescens*, *P. aeruginosa*	-	+	-	+	-	Va
H-4, S-3, S-4, S-6, S-7, SB-5, 15, 19	+	+	-	+	-	Vb

S-4, S-6, and S-7 which were isolated from cherry bud wood acquired from Sweden. Also included in group Vb were isolates H-4 and SB-5 from cherry trees near Salem, Oregon, as well as three isolates from the Plant Pathology farm at Corvallis.

Pathogenicity tests

Field tests

Based on the number of buds killed, the isolates were grouped into four classes; those in which 90-100% were killed, those in which 77-89% were killed, those in which 30-50% were killed, and those in which there was no statistically significant difference between those inoculated and the uninoculated controls (Table 3). All but two of the isolates that appeared to cause death of the inoculated buds were of group Ia of Lelliott et al. (1966) and were classified as P. syringae or P. morsprunorum. These two exceptions were isolate 48a and the isolate of P. aeruginosa. Both belonged to group Va and each had 11 of 35 buds killed, which was slightly higher than the controls.

None of the other phytopathogenic nomenspecies were pathogenic under these conditions, nor were any of the oxidase positive isolates except the two mentioned above.

The relationship of original host to virulence varied with the isolates. Pseudomonas syringae isolates from cherry ranged from highly virulent to avirulent, as did those from prune. The isolates from pear were either avirulent or only weakly virulent in this test. Isolate HPC (bean) and 912-S2 (almond) were highly virulent while LM-1 (lilac),

Table 3. Percent of inoculated cherry buds killed by *Pseudomonas* isolates.
Actual percentages in parentheses.

90-100%		77-90%		30-50%		≤ controls
GW-4	(100)	940-R	(86)	B-3	(49)	B-301
GW-9	(100)	GW-8	(86)	43-1	(40)	E-3
HPC	(100)	SC-4	(83)	S-30t	(34)	*P. glycinea*
GS-3S	(100)	B-299	(80)	P-2	(34)	*P. phaseolicola*
GG-2	(100)	MP	(77)	857-S2	(31)	*P. lachrymans*
919-S	(100)			48a	(31)	LM-1
SC-1	(100)			43-2	(31)	Ps 144
GG-1	(97)			*P. aerug.*	(31)	Syr D
Fr pr	(97)			P-3	(30)	7fS1
GW-13	(94)					all oxidase + isolates
912-S2	(94)					
B-3AS	(91)					

and Syr D (host unknown) were avirulent on the inoculated buds. Figure 3 shows an example of the type of results obtained.

Laboratory tests: Inoculation of green cherry fruits.

None of the oxidase positive isolates caused symptoms to develop on the cherry fruits. The three isolates of P. phaseolicola, SV-2, 21-B, and 9-B; the two isolates of P. morsprunorum, as well as the isolate of P. glycinea, caused small necrotic lesions and were given ratings of "one". Isolate 94-R, which, as previously indicated, was rejected as being P. syringae, also was rated in class "one". This was the only indication of pathogenicity in any of the tests conducted with this isolate. As shown in Table 4, most of the P. syringae isolates caused well developed necrotic lesions on the cherry fruits. 18 received a rating of "four", while six were rated "three". Figure 4 shows the results with selected isolates. The cherry fruits are apparently sensitive indicators of virulence as shown by the fact that Pseudomonas glycinea and P. phaseolicola, not normally cherry pathogens, both caused slight but detectable lesions on the fruits. On the other hand, the isolates of P. morsprunorum, normally pathogenic on cherry, caused only a slight reaction on the excised fruits.

Inoculation of green prune fruits.

Only the isolates of P. syringae and P. morsprunorum caused symptom development on the prune fruits. Neither the other oxidase negative nomenspecies nor any of the oxidase positive isolates caused any reaction. The prune fruits were not as sensitive to the bacteria as

Figure 3. Cherry tree inoculated with Pseudomonas isolates. Note the two branches on the right with all inoculated buds as well as the terminal buds killed. Other branches have some buds killed by the bacteria.

Table 4. Virulence ratings of *Pseudomonas* isolates when inoculated into green cherry fruits and incubated at 15° C. Rating scale of 0-4; 0 = no symptoms, 1 = small necrotic lesions, 4 = large dark sunken lesions, 2 and 3 = intermediate sized lesions.

RATING				
4	3	2	1	0
GW-4	GS-3S	7fS1	MP	all oxidase + isolates
GW-8	940-R	S-30t	B-299	
GW-9	SC-1	Syr D	94-R	
GW-13	SC-4		*P. glycinea*	
B-3	E-3		*P. phaseolicola*	
B-3AS	Ps-144			
GG-1				
GG-2				
43-1				
43-2				
912-S2				
919-S				
HPC				
P-2				
LM-1				
857-S2				
B-301				
SC-2				

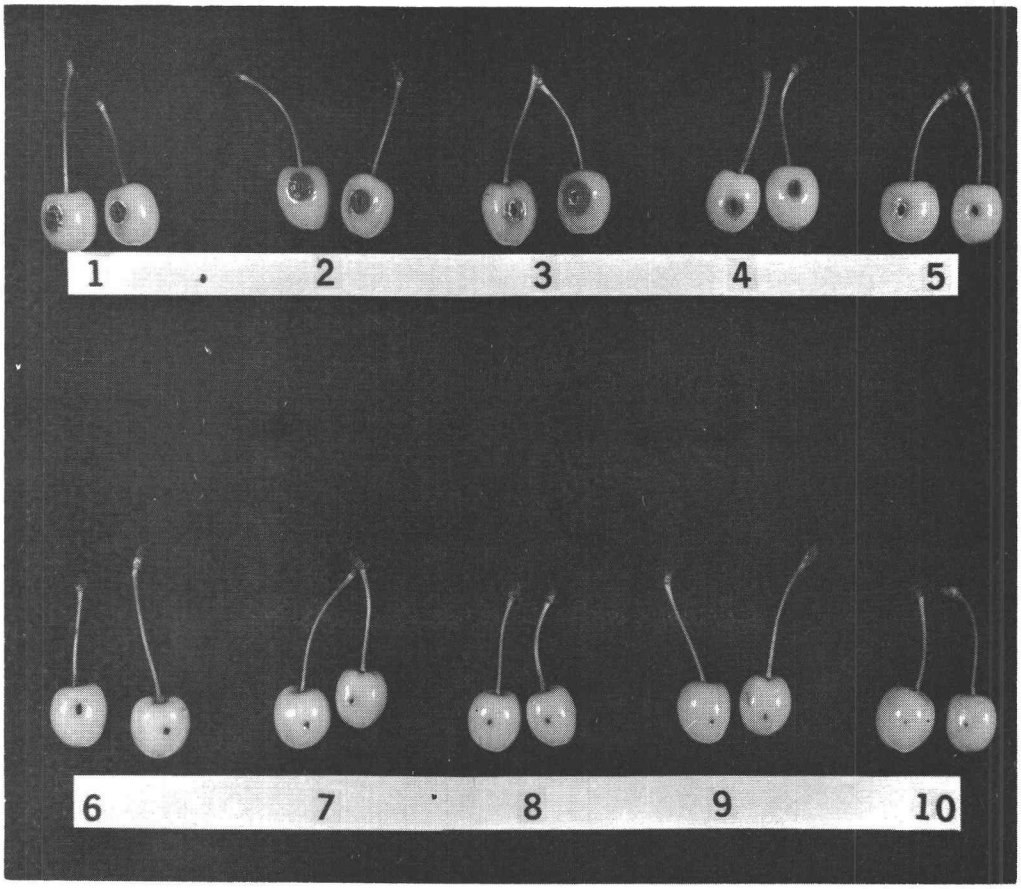


Figure 4. Typical reactions of Pseudomonas isolates in green cherry fruits.

1. B-3
2. 43-2
3. SC-2
4. B-301
5. P-2
6. 43-1
7. P. morsprunorum
8. P. phaseolicola
9. P. glycinea
10. control

cherry as indicated by the fact that only three P. syringae isolates were rated "two" or less on cherry, while thirteen of the isolates were rated "two" or less on prune (Table 5). In general there was agreement between the results on cherry and prune fruits even though the isolates were more evenly distributed among the rating groups on prune. The isolates that proved highly virulent on cherry were also rated highest on prune. Conversely those that showed relatively low virulence on cherry were low on prune. Exceptions to this were isolates SC-4 and Ps 144. Both were rated "three" on cherry and "one" on prune. These results are not consistent with the bud inoculations, where isolate SC-4 killed 83%, while Ps 144 was rated as non-virulent. Hence the rating of SC-4 on cherry fruits would agree more closely with the rating on bud kill than would the rating on prune fruits. On the other hand, the rating of Ps 144 on prune fruits would agree more with the bud kill than would the rating on cherry fruits. Some comparative results on prune are shown in Figure 5.

Inoculation of green pear fruits.

There was not as much variation in lesion size on pear fruits as on cherry and prune. Consequently the rating scale ranged from zero-three rather than zero-four as for cherry and prune (Table 6). Those rated as "zero" included P. morsprunorum, P. glycinea, P. phaseolicola, isolate 94-R and all of the oxidase positive isolates. All but five of the P. syringae isolates were rated "two". Again the results were reasonably consistent with those using cherry and prune in that isolates GG-2, 919-S, and HPC, rated "three" on pear, rated "four" on cherry and

Table 5. Virulence ratings of Pseudomonas isolates when inoculated into green prune fruits. Rating scale; 0-4, 0 = no symptoms, 4 = large dark sunken lesions. Other ratings intermediate in symptom expression.

RATING				
4	3	2	1	0
GW-4	GS-3S	7fS1	SC-4	P. glycinea
GW-8	B-3	GW-9	Ps 144	P. phaseolicola
GW-13	LM-1	43-1	S-30t	94-R
B-3AS		43-2	MP	oxidase + isolates
GG-1		SC-1	B-299	
GG-2		857-S2		
912-S2		940-R		
919-S		B-301		
HPC		E-3		
P-2				
Fr pr				

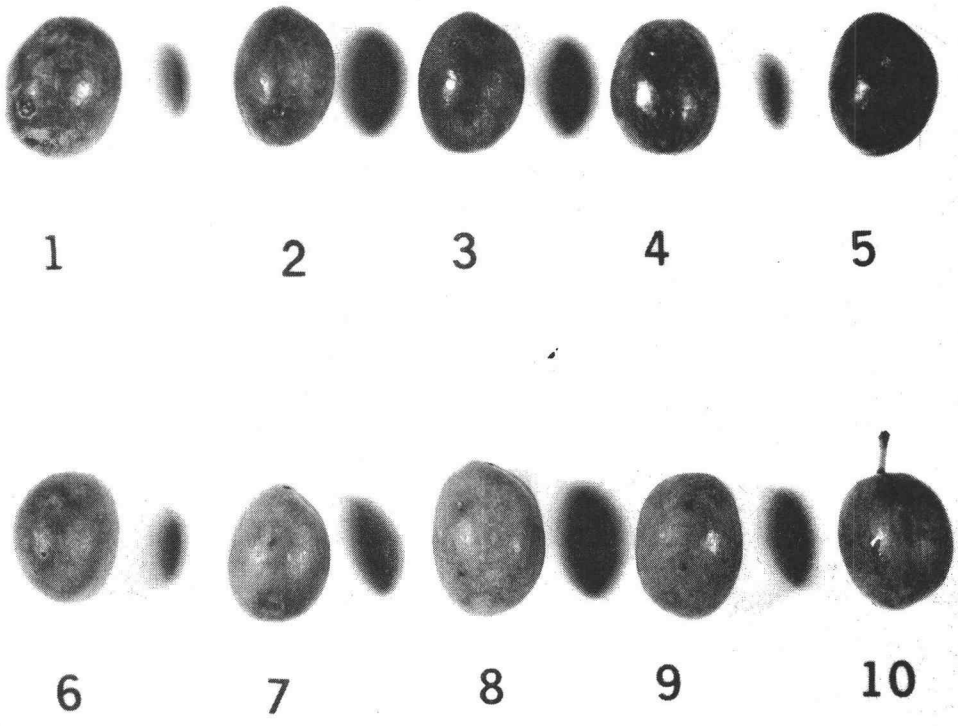


Figure 5. Typical reactions of Pseudomonas isolates in green prune fruits.

1. B-3
2. 43-2
3. GG-2
4. B-301
5. P-2
6. 43-1
7. P. morsprunorum
8. P. phaseolicola
9. P. glycinea
10. control

Table 6. Virulence ratings of Pseudomonas isolates when inoculated into green pear fruits. Rating scale; 0-3. 0 = no symptoms, 1 = small necrotic lesions, 3 = large dark necrotic lesion, 2 = intermediate lesions.

RATING				
3	2		1	0
GG-2	GW-4	P-3	S-30t	MP
919-S	GW-8	LM-1	Syr D	B-299
HPC	GW-9	857-S2		P. glycinea
	GW-13	B-301		P. phaseolicola
	B-3	SC-1		94-R
	B-3AS	SC-2		all oxidase + isolates
	GG-1	SC-4		
	GG-2	GS-3S		
	43-1	E-3		
	43-2	Ps 144		
	912-S2	7fS1		
	P-2	940-R		
		Fr pr		

prune, while S-30t and Syr D rated "one" on pear also rated "one on prune and "two" on cherry. Comparative results on pear fruit are shown in Figure 6.

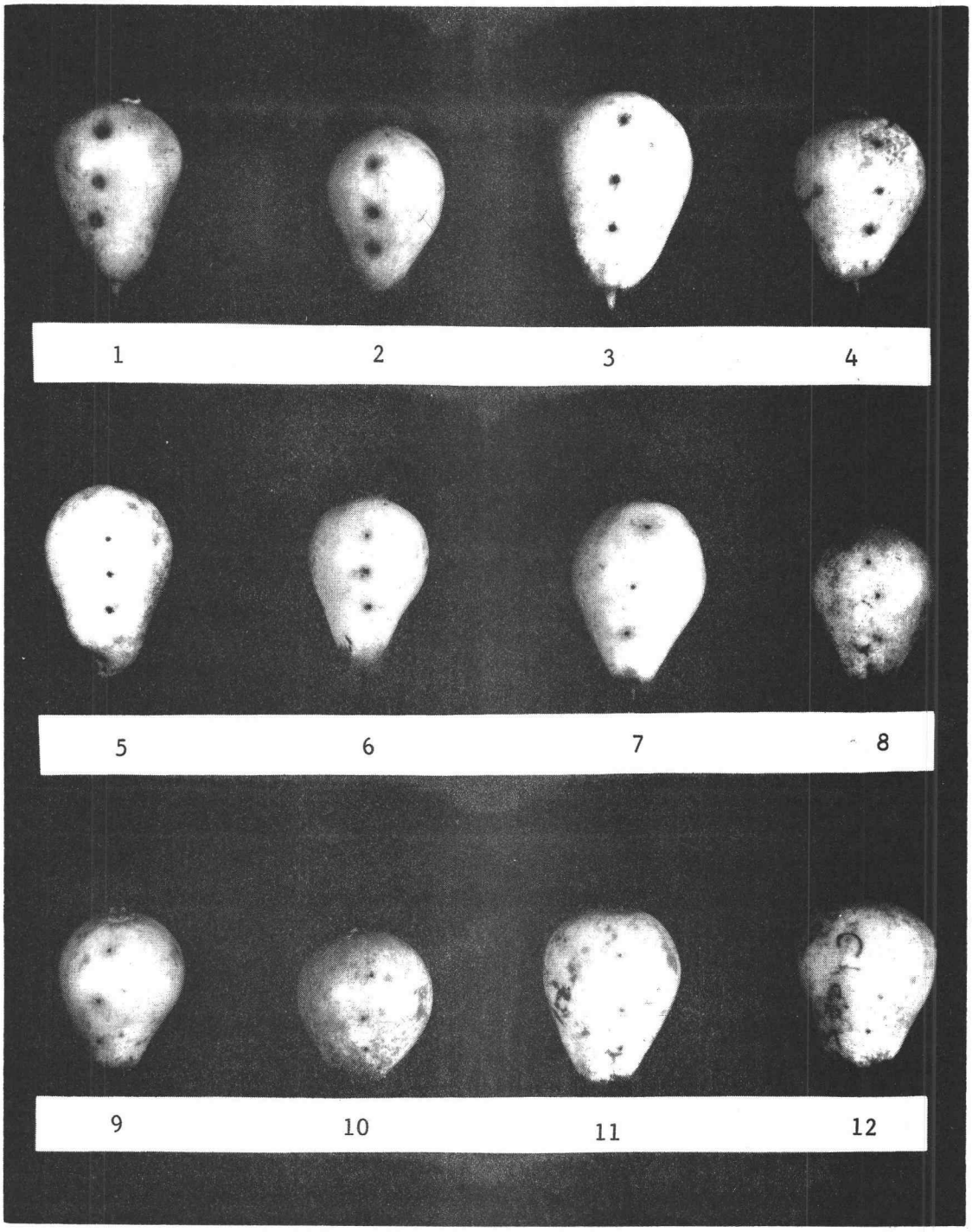
Protein electrophoresis

Soluble protein extracts separated into approximately 25 bands following electrophoresis. Small uncontrollable differences in migration velocities occurred in some tubes, but duplicates of the same extraction run at the same time produced identical patterns. In the solutions frozen for several months some bands did not stain as intensely as they did in fresher material. In addition, some bands lying close together did not separate as distinctly in older material as they did in fresher samples (Figure 7). Both the fresh and older extracts could be identified as having come from the same isolate, however. In a test run comparing samples of the same isolate cultured under different conditions (48 hours at 30° C vs 72 hours at 25° C) very similar patterns were also produced. The variation was no greater than that shown by samples frozen for different lengths of time.

As expected, the plant pathogens of LOPAT group Ia had a number of protein bands in common with the saprophytic isolates of groups IVa, Va, and Vb. Nevertheless, a clear distinction could be made between the protein patterns of the plant pathogens and the saprophytes. Each of the pathogenic nomenspecies had patterns similar to the others, with most of the bands matching those in each of the other species. Differences in the patterns were great enough, however, to enable a clear separation of these nomenspecies. A notable feature of the

Figure 6. Typical reactions of Pseudomonas isolates in green pear fruits.

1. 919-S
2. GG-2
3. HPC
4. B-3
5. E-3
6. B-301
7. 43-1
8. SC-2
9. P-2
10. S-30t
11. *P. morsprunorum*
12. control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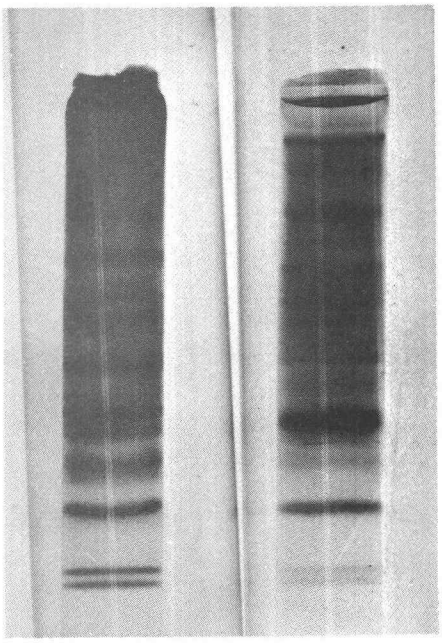

Figure 7. Comparison of two gels from the same extract of Pseudomonas phaseolicola kept frozen for different lengths of time. Left, run on 8-13-69, one week after extraction. Right, run on 12-2-69. Note that in some cases what appears as two narrow bands on the left appears as a single wide band on the right.

patterns was the presence of a broad, densely staining band just behind the two dark frontal bands in gels from P. morsprunorum, P. glycinea, and P. phaseolicola which was absent in P. syringae and P. lachrymans. Thus these two groups could be distinguished on the basis of the presence or absence of this band alone. Within these groups the species could be separated by the presence of other non-homologous bands in each species. Densitometer tracings of these species are shown in Figure 8.

All three isolates of P. phaseolicola had the same patterns (Figure 9). Likewise the two isolates of P. morsprunorum were identical. All of the named isolates of P. syringae as well as those isolated locally and assumed to be P. syringae on the basis of the LOPAT characteristics and pathogenicity tests had very similar band patterns. There were some differences among isolates but these differences were not as great as those between species. They differed mostly in relative intensity of some of the bands. Differences could not be correlated with original host, geographical location, pathogenicity, or in those cases where it was known, the serological type. Representative densitometer tracings of isolates from different hosts are shown in Figures 10-13. Isolate 94-R, which was oxidase negative but not pathogenic had a pattern very similar to the oxidase negative plant pathogens but was distinct from each of the species being compared (Figure 8). It was also distinct from the oxidase positive saprophytes.

The saprophytic isolates were divided into several groups on the basis of band patterns. The two isolates placed in LOPAT group IVa, each had different patterns by which they were easily separated from

Figure 8. Densitometer tracings of protein patterns of different Pseudomonas species. 1. P. glycinea, 2. P. phaseolicola, 3. P. morsprunorum, 4. P. syringae, 5. P. lachrymans, 6. isolate 94-R, 7. P. fluorescens, 8. P. aeruginosa. Note the prominent band, third from the left in the first three species that distinguishes them from the others. (+) and (-) denote anode and cathode ends of gels respectively, and arrow denotes migration direction of proteins through the gels.

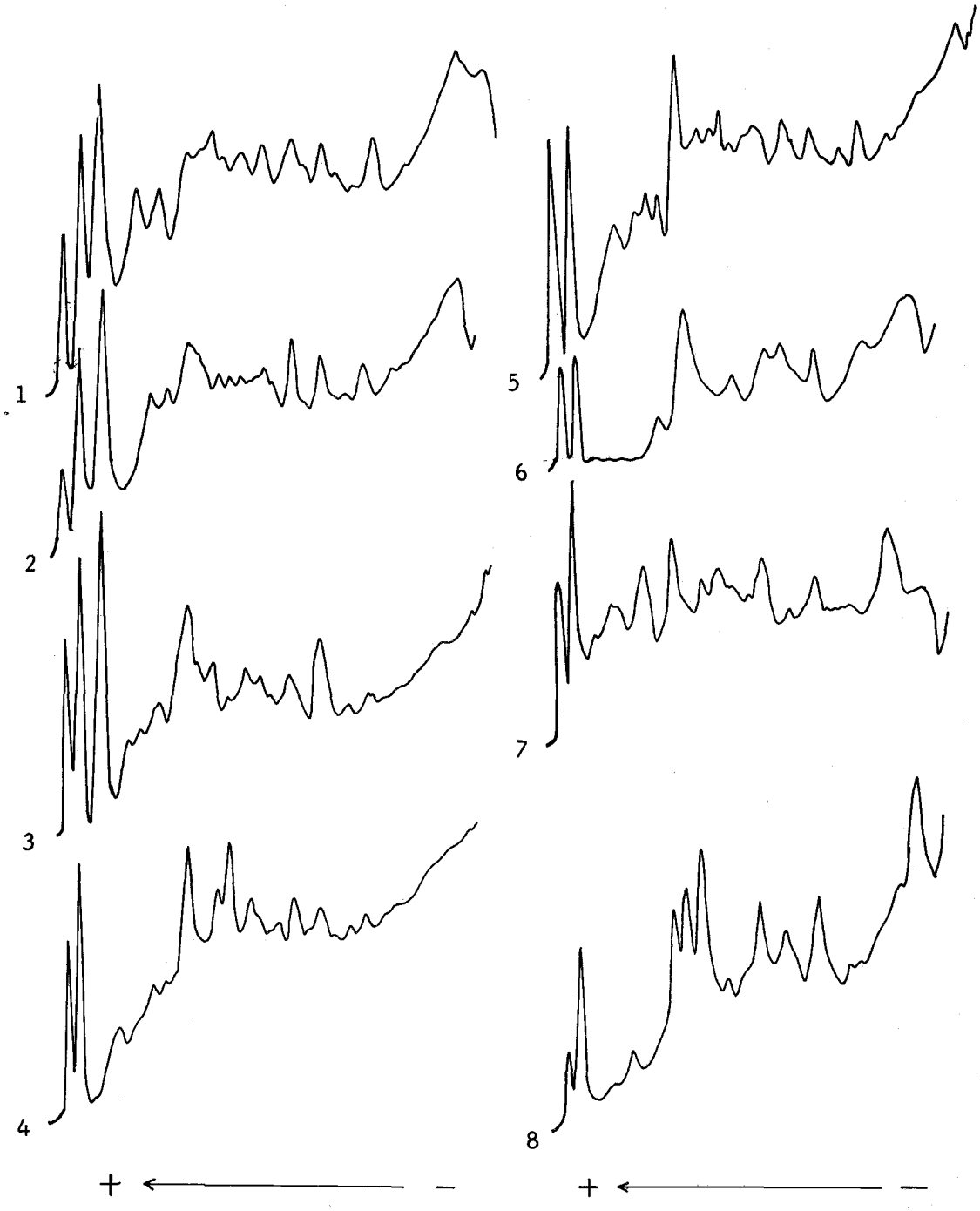
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Figure 10. Densitometer tracings of protein patterns of Pseudomonas syringae isolates from different hosts. Top to bottom: Fr. pr., prune; GW-13, cherry; 912-S2, almond; HPC, bean; LM-1, lilac; E-3, pear; B-3AS, peach.

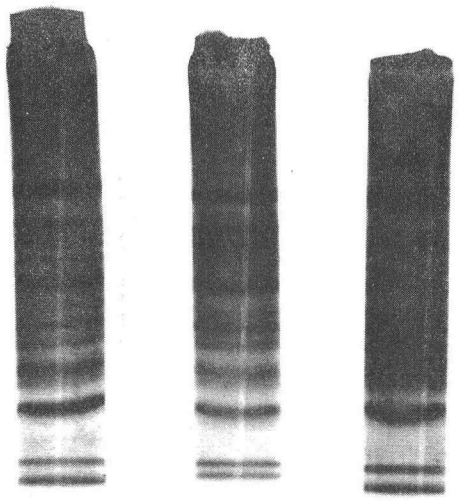


Figure 9. Comparison of three isolates of Pseudomonas phaseolicola showing virtually identical protein band patterns in all three. Left to right, SV-2, 21-B, 9-B.

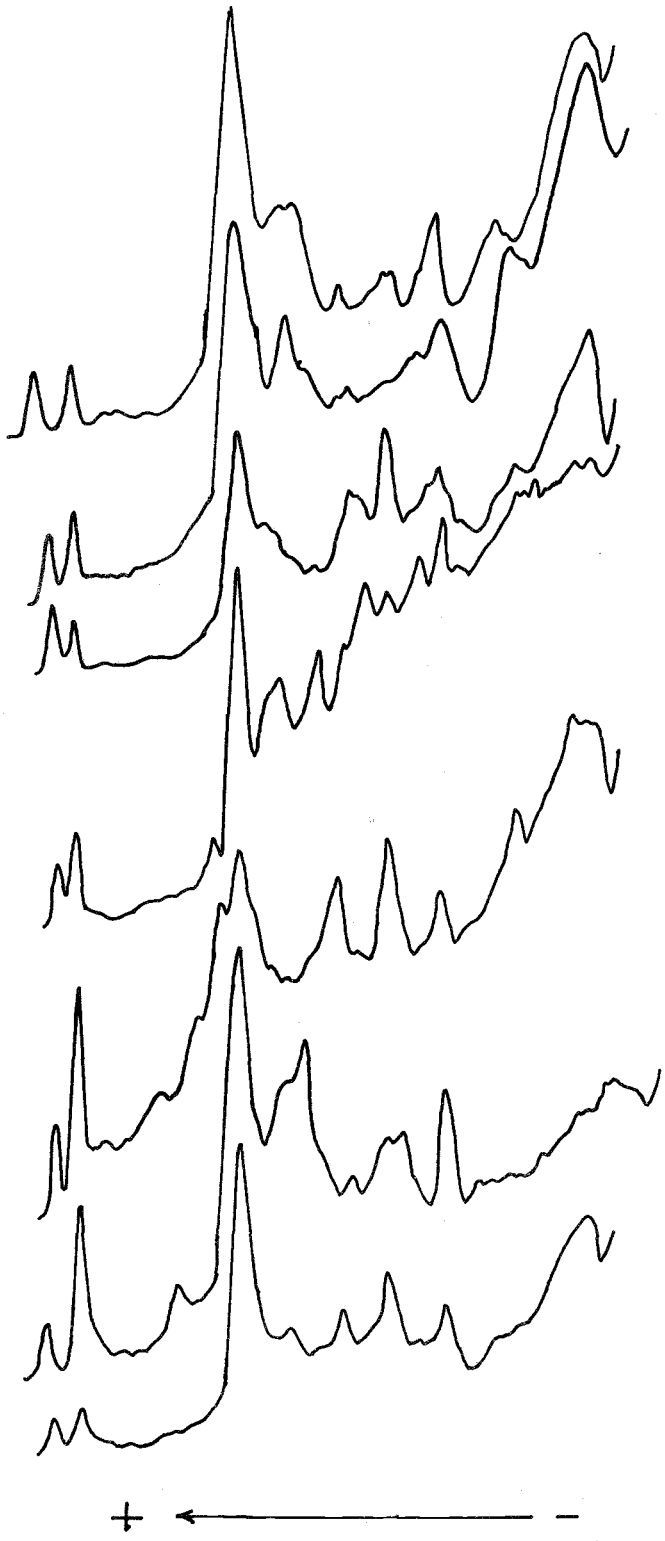


Figure 11. Densitometer tracings of protein patterns of Pseudomonas syringae isolates from pear. Top to bottom; S-30t, P-3, P-2, B-301.

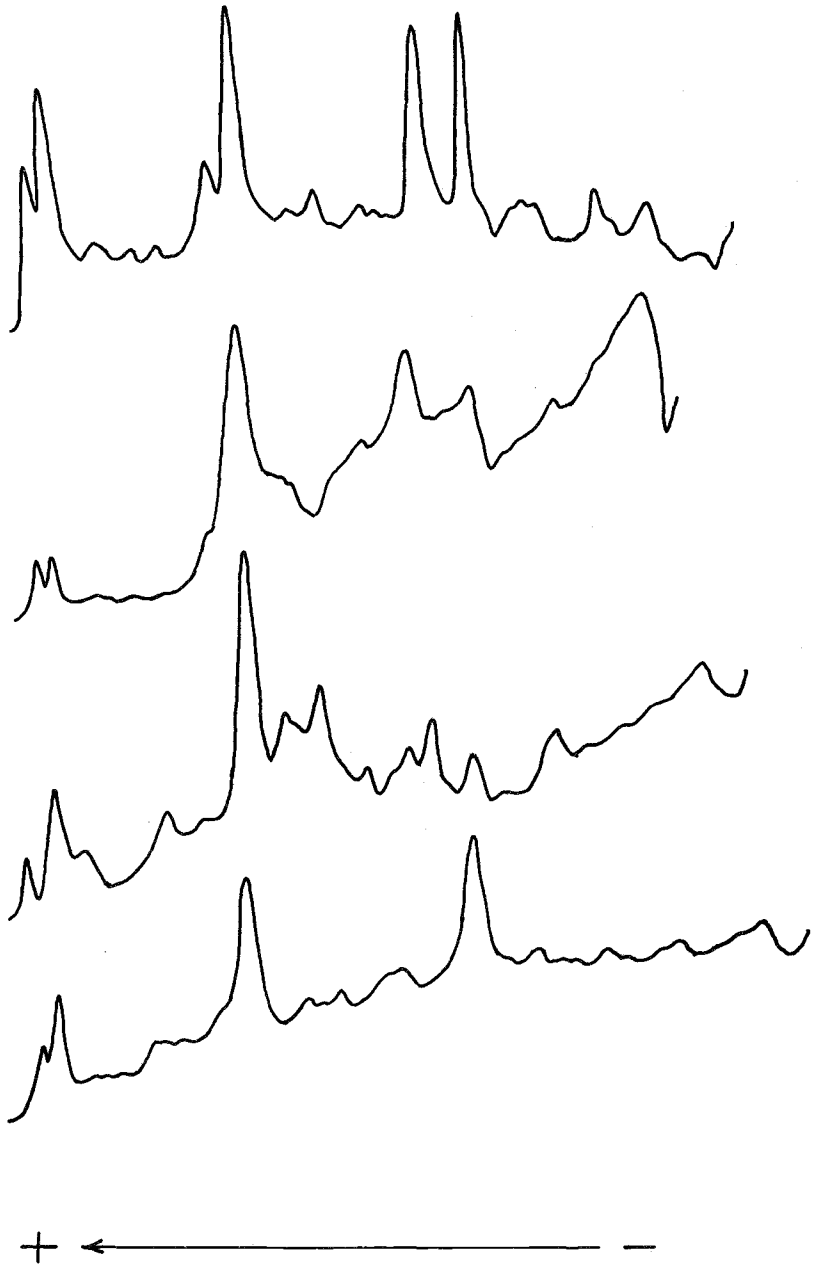


Figure 12. Densitometer tracings of protein patterns of Pseudomonas syringae isolates from prune. Top to bottom; 857-S2, GS-3S, GG-1, GG-2.

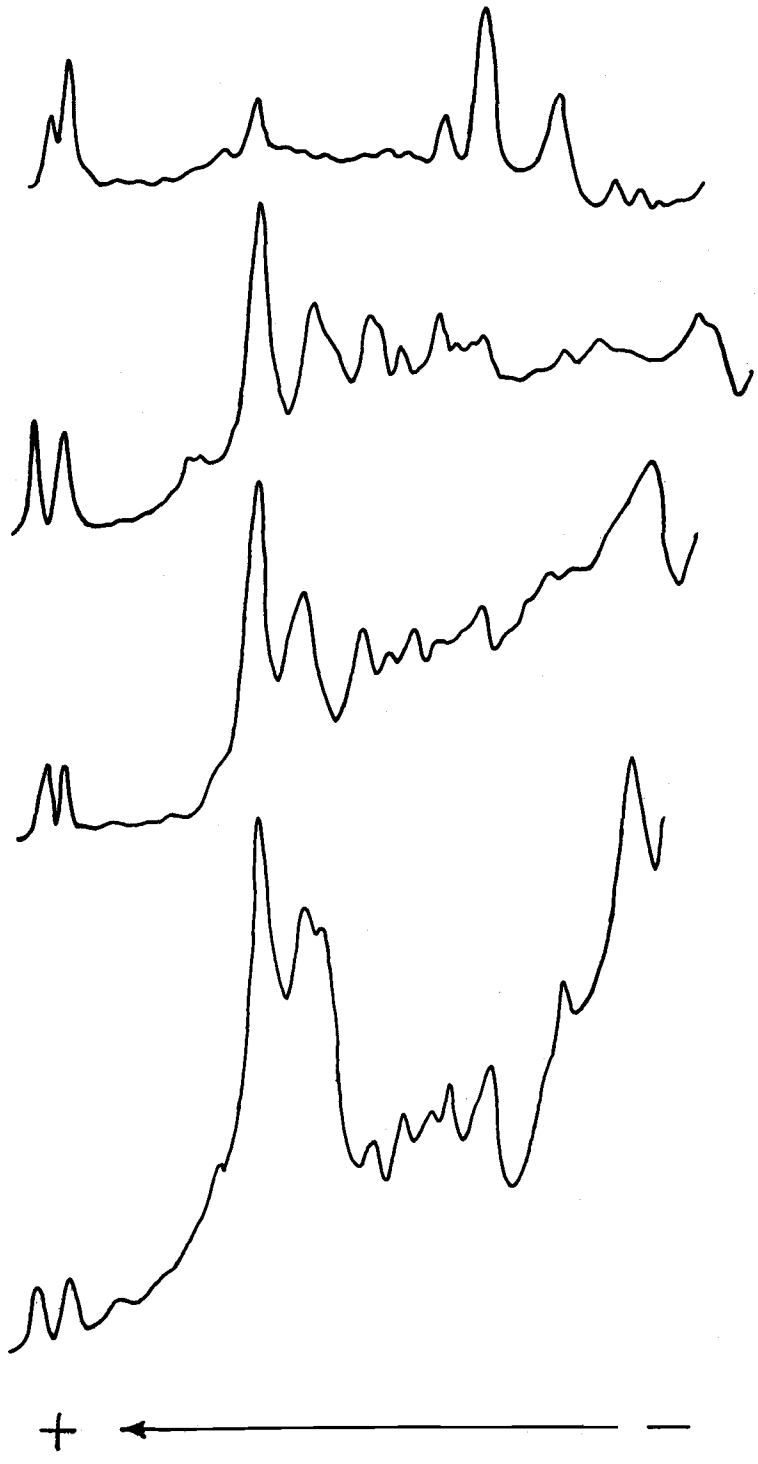
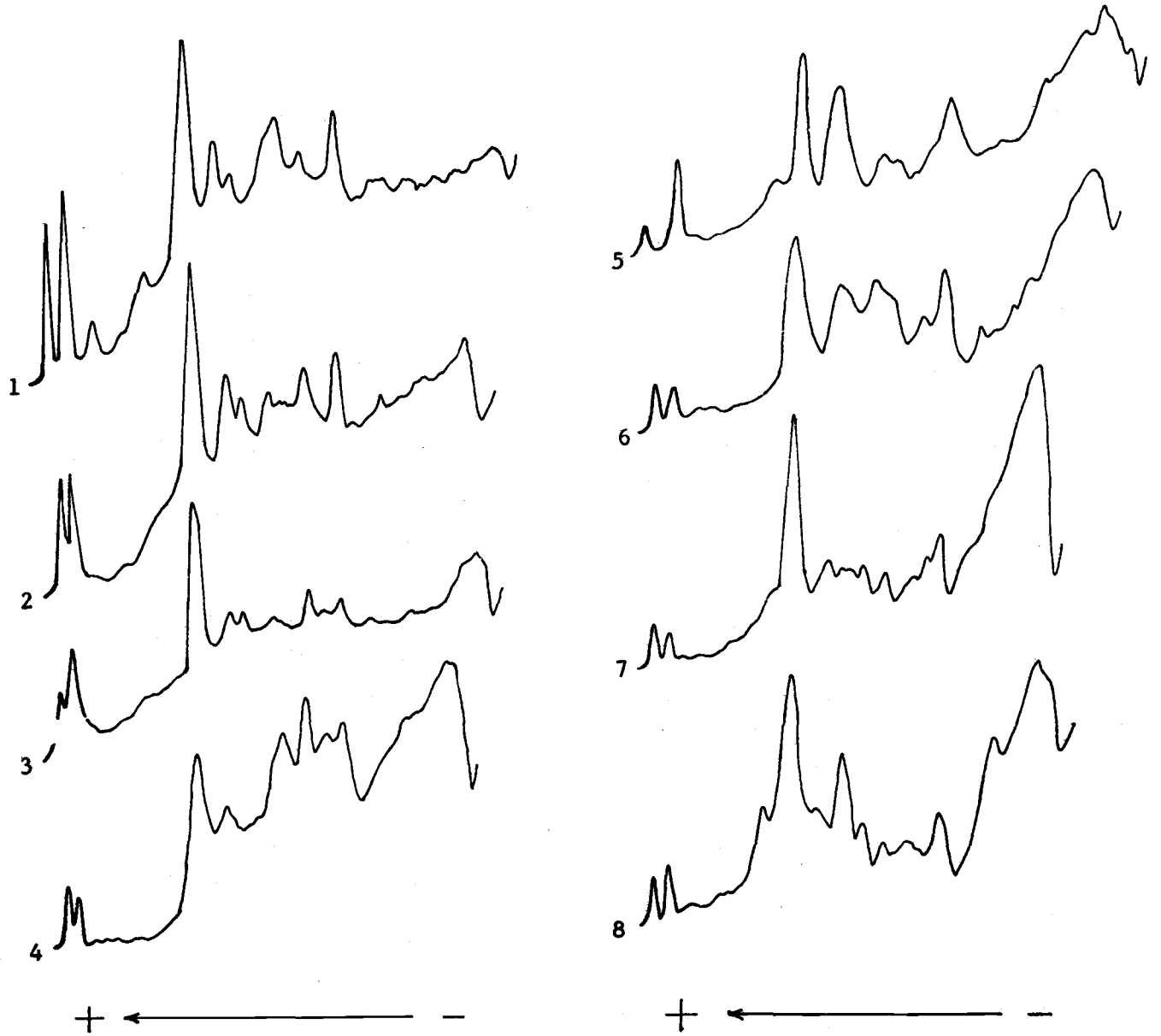


Figure 13. Densitometer tracings of protein patterns of Pseudomonas syringae isolates from cherry.

1. 940-R
2. 43-1
3. 43-2
4. Ps 144
5. GW-4
6. GW-9
7. SC-1
8. SC-4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one another. Those isolates placed in LOPAT group Va were divided into three groups based on similarity of band pattern (Table 7).

The majority of those in group Va had patterns similar to that of the P. fluorescens isolate. Five additional isolates had patterns similar to each other but distinct from the P. fluorescens group. The isolate of P. aeruginosa and one other isolate constituted a third group based on band pattern (Figure 13).

Subgroups within LOPAT group Vb could also be distinguished on the basis of band pattern. (Table 7). Isolates S-3, S-4, SB-5, and 15 had similar patterns, constituting one group. Isolates S-6 and S-7 had a different pattern (Figure 14). Isolates H-4 and 19 were not similar to each other nor like any of the others. Thus among the oxidase positive saprophytes nine different groups based on similarity of band pattern could be recognized. Unfortunately, since this study was primarily concerned with the pathogenic species, there were not enough named saprophytic isolates included for an adequate comparison of these isolates.

Table 7. Grouping of oxidase positive Pseudomonas isolates based on similarities of protein band patterns.

LOPAT group	PROTEIN BAND GROUP			
	1	2	3	4
Group IVa	20-1	H-2C		
Group Va	P. fluorescens	SB-6	P. aeruginosa	
	8-1-2	14	38-20-1	
	8-5-1	47-48-1		
	8-19-3	86-39		
	8-19-4	86-49		
	8-40-8			
	48a			
Group Vb	19	H-4	S-6	SB-5
			S-7	15
				S-3
				S-4

Figure 14. Densitometer tracings of protein patterns of oxidase positive pseudomonads of LOPAT group Va. Left, protein pattern group 1, right, group 2.

1. 8-1-2
2. 8-5-1
3. 8-19-3
4. 8-19-4
5. 48a
6. 47-48-1
7. 86-39
8. SB-6
9. 86-49
10. 14

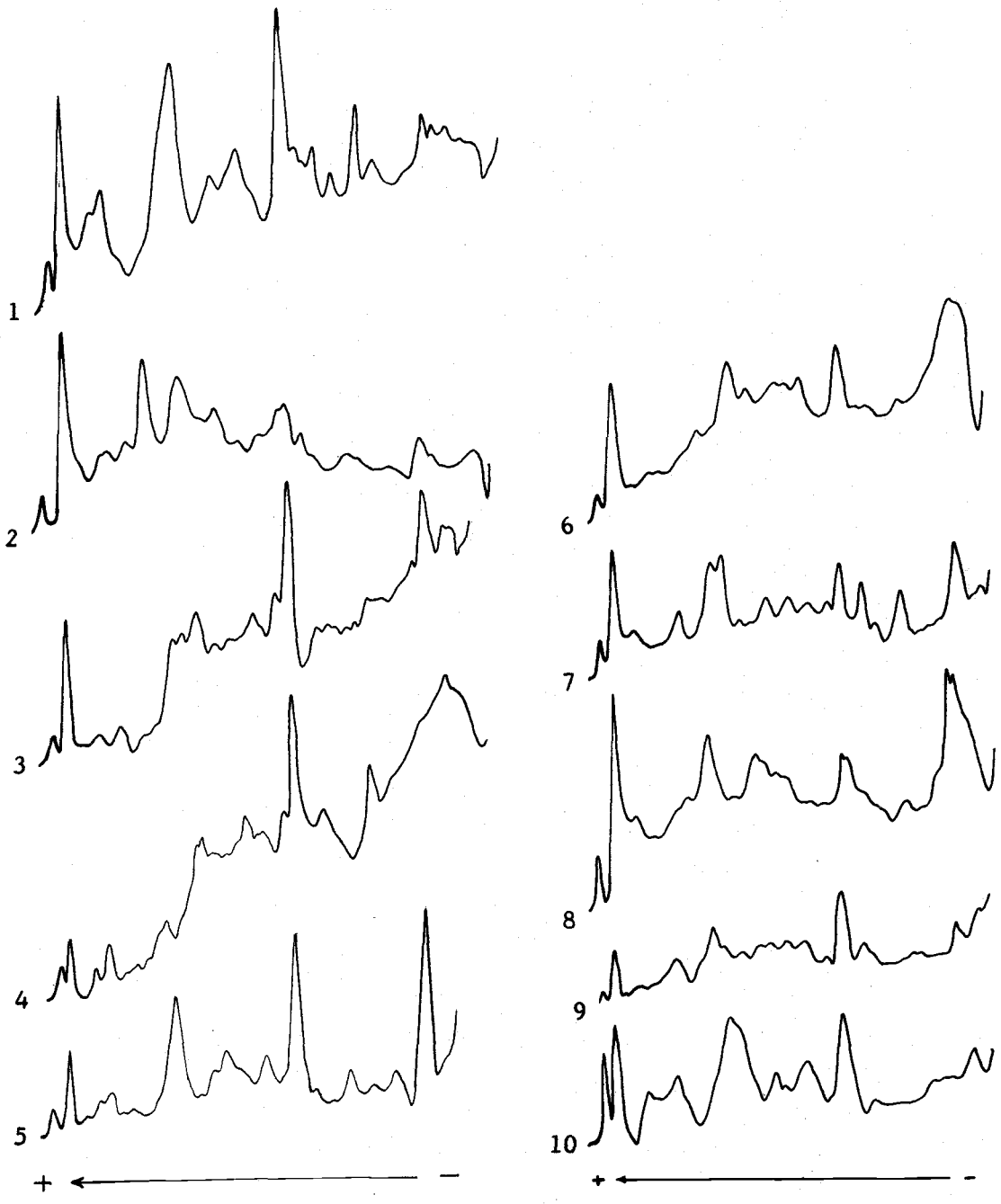
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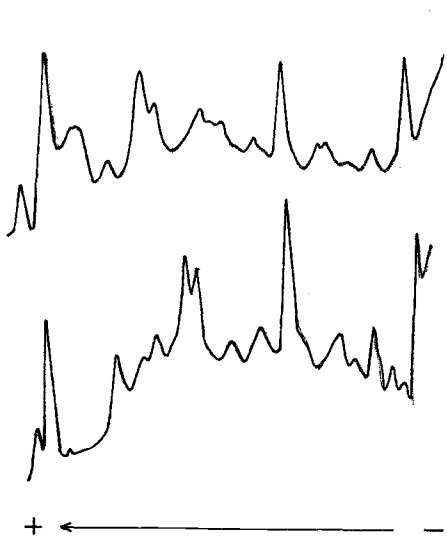
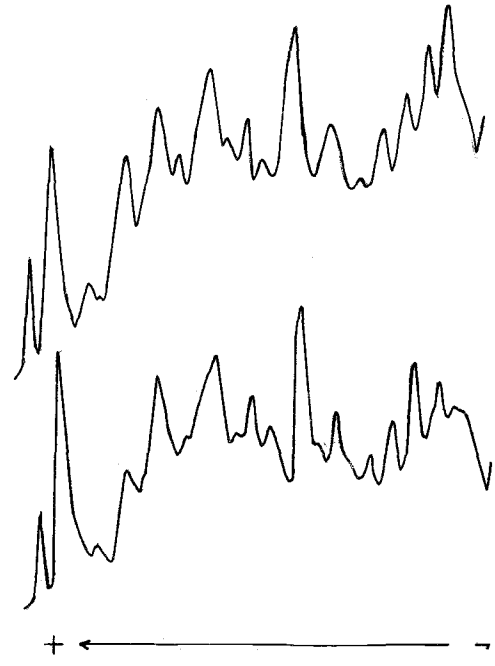
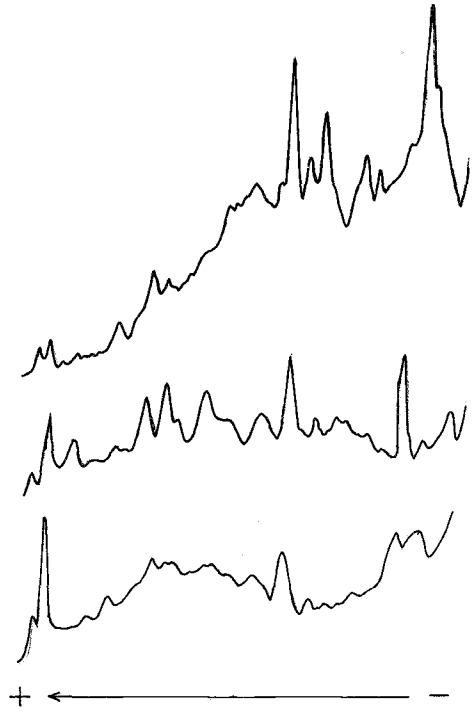
Figure 15. Densitometer tracings of protein patterns of oxidase positive pseudomonads of LOPAT group Vb. Top left; isolates 19 and H-4 of protein groups 1 and 2 respectively; top right, S-6 and S-7 of protein group 3; bottom, S-4, 15, and SB-5 of protein group 4.







DISCUSSION

The work reported here verifies that of others (Otta and English, 1971; Lelliott et al., 1966; Garrett et al., 1966) in that the plant pathogenic, fluorescent pseudomonads can readily be separated from the saprophytic forms by means of the oxidase test. None of the oxidase positive isolates except the isolate of P. aeruginosa and isolate 48a gave indications of being pathogenic. Both of these had slightly higher rates of bud kill than the controls in the orchard tests, but in both cases it was found that at least one branch of the three inoculated with each isolate was adjacent to a branch having severe natural infection. Spread of bacteria from these branches may have accounted for the apparent pathogenicity. Under laboratory conditions none of the oxidase positive isolates caused symptom development.

Otta and English (1971) report that Lovell peach seedlings make a suitable host for screening for pathogenic isolates of P. syringae regardless of their original host. This is because the peach is susceptible regardless of the source of the pathogen. Our results indicate that green cherry fruits or green prune fruits are also suitable screening hosts. All of the isolates considered to be P. syringae or P. morsprunorum caused symptom development on these hosts. Since P. glycinea and P. phaseolicola isolates caused the same type of lesions on cherry fruits as did P. morsprunorum, the cherry fruits could not be used to separate these nomenspecies. P. glycinea and P. phaseolicola were not pathogenic on the prune fruits, however.

Bacteria infecting cherries and plum in England (Crosse, 1968)

and Switzerland (Burki, 1968) are referred to as P. morsprunorum, while those from other hosts are classed as P. syringae. Burki (1968) and Crosse (1968) were able to distinguish between the two on the basis of host reaction. P. syringae would cause lesions when inoculated into green pear fruits but P. morsprunorum would not. Our results are in agreement in that the two isolates of P. morsprunorum caused no lesions in pear but did cause small ones in prune and cherry fruits. All of the P. syringae isolates caused symptom development in the pear as well as the prune and cherry fruits.

The value of electrophoretic data depends upon the procedure and methods used in interpreting the results. This has been emphasized by Shipton and Fleischmann (1969) and Shipton and McDonald (1970). For example, uncontrollable variation in migration rates between tubes presents a problem when attempting to determine which bands constitute homologues (i.e., have the same migration velocities). This is especially critical when comparing gels from separate runs. A number of workers (Smith and Powell, 1968; Gottlieb and Hepden, 1966; Meyer and Renard, 1969) have used E_f values for different protein bands as a basis of comparison. In our work the E_f values for a number of gels were calculated, but the results were completely unsatisfactory. Based on E_f values there was greater variation among isolates of P. syringae than between P. syringae and the other phytopathogenic nomenspecies. Evaluation of the results by the method of Johnson et al. (1967), comparing 1:1 mixtures of paired extracts with each member of the pair in order to determine homologous bands, was found to give more satisfactory results.

Our results are in general agreement with others in indicating that a clear separation of organisms at the nomenspecies level can be made by means of gel electrophoresis. Therefore this method should be of value as a relatively rapid and simple means of identification of species of Pseudomonas. Other methods such as serology must be utilized for determinations at the sub-specific level, however.

Electrophoretic data are significant in the light of other data concerning relationships among the species tested. Some maintain that P. syringae and P. morsprunorum constitute a single nomenspecies. This view is supported by serological data in which Lovrekovich et al. (1963) and Otta and English (1971) found that certain isolates of the two species were of the same serotype. Burki (1968) on the other hand, found that while the somatic antigens of the two were the same, a distinction could be made between them on the basis of flagellar antigens. Our data show that each of the five species tested, including P. morsprunorum, differed from all of the others by the presence of five to six non-homologous bands between each pair of species. Our electrophoretic data support the view of Burki (1968) and Crosse (1968) that P. morsprunorum is an acceptable nomenspecies distinct from P. syringae. The variation in band patterns among the various P. syringae isolates is also in agreement with serological data which shows the heterogeneity of the species, even though serological types and band patterns could not be correlated.

Electrophoretic data confirm the close relationships reported for the oxidase negative plant pathogens as well as their distinction from the saprophytic species (Misaghi and Grogan, 1969). There were about

twice as many non-homologous bands between the saprophytic and pathogenic species as there were between the different pathogens.

Based on the close biochemical relationships demonstrated by others and confirmed here by electrophoretic patterns, we support the placement of P. syringae, P. morsprunorum, P. glycinea, P. lachrymans, P. phaseolicola, and probably the other oxidase negative plant pathogenic pseudomonads into a single taxospecies. However the distinction between nomenspecies within this group is very useful to plant pathologists, and can be made by means of gel electrophoresis of soluble proteins.

Insufficient named saprophytic isolates were used in this study for an adequate comparison, therefore it was not possible to group the oxidase positive isolates into nomenspecies. The grouping of the isolates into LOPAT subgroups based on band patterns is only tentative. In most cases the members of a group had band patterns that were more nearly like the other members of that group than like members of the other groups, but were not identical. Only isolates S-6 and S-7 had virtually identical patterns. A more detailed study of these oxidase positive isolates would likely reveal a greater variation than the present grouping suggests.

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