BACTERIAL BLIGHT OF *VICIA SATIVA*: AETIOLOGY OF THE DISEASE AND IDENTIFICATION OF THE PATHOGEN

By R. N. Allen,* A. C. Hayward,† W. J. Halliday,† and Jean Fulcher†

[*Manuscript received September* 15, 1969]

Summary

A serious new disease of vetch in the north coast area of New South Wales was shown to be caused by *Pseudomonas stizolobii* (Wolf) Stapp. The pathogen, recorded in Australia for the first time, was able to infect eight other species of legumes in glasshouse tests. Field diseases of *Trifolium repens* and *Mucuna* sp. caused by this organism were also recorded and isolates obtained from these sources were found to be pathogenic to vetch.

The bacterium was identified by comparison with two authentic cultures of *Ps. stizolobii* on the basis of cultural, physiological, and serological properties. There was evidence of serological heterogeneity in the Australian isolates.

I. Introduction

A serious leaf and stem disease of Golden Tares, a cultivar of common vetch (*Vicia sativa* L.), occurred at Wollongbar, N.S.W., in 1961, but its cause was not established. It occurred in this area in the following seasons, and also in the valleys of the Nambucca, Macleay, and Manning Rs., N.S.W., and was most severe following wet autumn weather. It was known variously as black stem rot, wilt, and decline, but is better referred to as bacterial blight.

The symptoms were unlike those of any disease previously described on vetch. Field symptoms developed at any stage of growth after the plants were about 3 weeks old, and often appeared near wounds. The lesions were deep red to black in colour, necrotic, linear, and limited by the stem veins. They were most severe on the lower parts of the older stems, causing these stems to die progressively from the base. Leaf lesions also occurred, usually close to stem lesions, and were deep red to black, circular, and surrounded by a chlorotic zone (Figs. 1 and 2).

When placed in water, bacteria oozed from the diseased cortical and epidermal tissue. Preliminary cultural studies of the bacterial isolates suggested that they were *Pseudomonas stizolobii* (Wolf) Stapp. This organism has previously been recorded in the United States of America on velvet bean (*Mucuna* sp.) and white clover (*Trifolium repens* L.) (Wolf 1920; Burkholder 1957); in Zambia on velvet bean

* Agricultural Research Station, Department of Agriculture, Wollongbar, N.S.W. 2480.

† Department of Microbiology, University of Queensland Medical School, Herston, Brisbane, Qld. 4006.

(Hayward, unpublished data), and in Rhodesia on *Bougainvillea* spp. (Rothwell and Hayward 1964). The Rhodesian strain was reported to be non-pathogenic to velvet bean, and thus differed from the other strains. In Australia, a bacterial leaf spot disease of velvet bean was recorded at Grafton, N.S.W., as *Bacterium* sp. (? *stizolobii*) in 1938 (Hynes *et al.* 1941) and although the identity of the pathogen was

Figs. 1 and 2.—Stem, petiole, and leaf lesions on vetch (Fig.1) and symptoms on older shoots (Fig. 2) caused by *Ps. stizolobii*.

not confirmed, the symptoms on velvet bean leaves,* artificially inoculated with bacteria from the field specimens, are identical with those caused by *Pseudomonas stizolobii*. The field symptoms occurred on plants grown from seed imported from the United States of America.

* These leaves are now filed in the Biology Branch Herbarium of the New South Wales Department of Agriculture (Ref. No. DAR716).

II. Aetiology

(*a*) *Methods*

The bacteria were isolated by standard methods (Dowson 1957, p. 27) on PYE agar containing glucose* after incubation at 28°C. Inoculum for pathogenicity tests was prepared, by suspending in 25 ml sterile water, bacteria from a 2-day-old smear plate culture of isolate 0268a (Table 1) on potato dextrose agar† after incubation at 25°C.

Eleven species of leguminous plants, including vetch (Table 2), were grown in clay loam sterilized with methyl bromide. The plants were kept in a humid chamber for 24 hr before inoculation, and some leaves and stems were wounded by a series of pin-pricks immediately before the plants were sprayed with inoculum. Plants sprayed with water were used as controls. After inoculation the plants were placed in a humid atmosphere for 24 hr at 21°C/25°C night/day cycle. After preliminary experiments involving one or more of the test species, inoculations of all test species were conducted on two separate occasions. Pathogenicity tests of isolates 0268c and 0300 (Table 1) were conducted on the test plants (Table 2) in separate experiments. Pathogenicity tests of isolates 0344a, 0353, and 0364 were conducted on vetch and velvet bean only.

(*b*) *Results*

Isolates of bacteria were obtained from fresh tissue of vetch, velvet bean, and white clover (Table 1). Circular, low convex, non-pigmented, translucent to opaque colonies, which were visible macroscopically after 36 hr incubation, grew on the isolation plates. Other types of colonies were rarely encountered. Attempts to isolate the

Table 1

ORIGINS OF BACTERIAL ISOLATES MENTIONED IN THE TEXT

Unless otherwise stated all localities are in New South Wales

Isolate Accession No.	Origin
0268a	Stems of *Vicia sativa* collected at Wollongbar in April, 1967
0268c	Leaves of *V. sativa* collected at Wollongbar in April, 1967
0292	Reisolated from *Mucuna* sp. artificially inoculated with isolate 0268a
0295	Reisolated from *V. sativa* artificially inoculated with isolate 0268c
0296	Reisolated from *Mucuna* sp. artificially inoculated with isolate 0268c
0300	Material of *V. sativa* collected at Mullumbimby in July, 1967
0344a	Leaves of *Mucuna* sp. collected at Broadwater in March, 1968
0353	Leaves of *Trifolium repens* collected at Kyogle in May, 1968
0364	Leaves of *T. repens* collected at Dorrigo in February, 1969
NCPPB* 450	*Pseudomonas stizolobii* isolated from *T. repens* in 1954 by W. H. Burkholder, Ithaca, New York
NCPPB 451	*Pseudomonas stizolobii* isolated from *T. pratense* in 1955 by W. H. Burkholder, Ithaca, New York

* National Collection of Plant Pathogenic Bacteria, Plant Pathology Laboratory, Harpenden, Herts., England.

bacterium from lesions on dried plant material stored for 5–13 months at room temperature were unsuccessful.

* Difco bacto peptone, 10·0 g; Difco yeast extract, 5·0 g; sodium chloride, 5·0 g; Difco bacto agar, 15·0 g; distilled water, 1 litre; pH to 7·2. Glucose solution was sterilized separately at 15 lb/in^2 for 30 min, and added to give a final concentration in the medium of 1%.

† Boiled water extract of 200 g chopped potato tubers; dextrose 20 g; agar 12 g; distilled water 1 litre; sterilized at 15 lb/in^2 for 20 min.

The symptoms of wound and stomatal infection on the 11 test species inoculated with isolate 0268a are presented in Table 2. Symptoms similar to those incited by isolate 0268a developed on the test plants inoculated with other isolates. Whereas wound infections developed quickly on most susceptible species, wounding did not

TABLE 2

SYMPTOMS CAUSED BY *PSEUDOMONAS STIZOLOBII* ON VARIOUS LEGUME HOSTS FOLLOWING ARTIFICIAL INOCULATION IN THE GLASSHOUSE

Plant Species	Stomatal Infection	Wound Infection
Vigna sinensis Endl. cv. Poona	Nil	Nil
Glycine max Merrill cv. Semstar	Nil	Nil
Dolichos lablab L. cv. Rongai	Nil	Brown necrosis on primary and trifoliate leaves 2–3 days after inoculation
Medicago sativa L. cv. Hunter River	Small brown spots on some leaves after 25–30 days	Brown necrosis on leaves and stems after 15–20 days
Phaseolus vulgaris L. cv. Redlands Greenleaf	Nil	Brown necrosis on first trifoliate leaf after 14 days, but not on the primary leaves or on the second trifoliate leaf which had just emerged at the time of inoculation
Vicia faba L.	Small red spots on some leaves after 25–30 days	Red necrosis on leaves and stems after 24 hours. Stem lesions with water-soaked halo
Trifolium repens L. cv. Ladino	Small brown necrotic spots on a few leaves after 30 days	Brown to black necrosis on leaves and petioles after 6 days, and on stolons after 21 days
Trifolium pratense L. cv. New Zealand Red	Nil	Brown necrosis on leaves and petioles after 4 days. Some leaf lesions became black, expanded, and developed a narrow yellow halo after 15–20 days
Vicia sativa L. cv. Golden Tares	A few spots, 1–2 mm diameter, with diffuse yellow halo after 20 days	Red necrosis on leaves and stems after 2 days. Stem lesions caused chlorosis and collapse of shoots after 15–20 days
Trifolium subterraneum L. cv. Clare	Black lesions, 1–2 mm in diameter, on many leaves after 15–20 days	Brown to black necrosis on leaves and petioles after 4 days. Petiole lesions caused leaf chlorosis
Mucuna sp.	Black, water-soaked spots on leaves after 6–10 days, developing into circular to angular spots with a distinct yellow halo	No infection at stem wounds. Some leaf wounds developed symptoms similar to stomatal infection, but some leaf wounds were not infected

appear to facilitate infection on velvet bean leaves or stems. Symptoms originating from stomatal infections were generally slow to develop, except on velvet bean leaves. The reactions of the plants agree closely with the results of Burkholder (1957), and differed only in the reactions of *Medicago sativa* and *Dolichos lablab*, which were wound-inoculated in Burkholder's tests and found to be non-reactive.

III. Identification of the Pathogen

(a) *Methods*

Two authentic cultures of *Pseudomonas stizolobii* were obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England, and compared in cultural and physiological tests with isolates 0268a, 0268c, 0292, 0295, 0296, and 0300 (Table 1). References to standard tests are given in Table 3.

Antibiotic sensitivity tests were carried out using Oxoid Multodisks code Nos. 11–14c, U1, S1, and 11–15F. Gentamicin Bacto sensitivity disks were supplied by Difco laboratories. The disks were applied to the surface of well-dried PYE agar plates which had been inoculated over the whole surface with 0·2 ml of a distilled water suspension of the test bacterium.

Agglutination reactions between bacterial suspensions of isolates NCPPB 450, NCPPB 451, 0300, 0344a, 0268a, and 0292 and rabbit antisera were examined. Antisera were prepared using cells from slope cultures that were harvested in saline, heated at 100°C for 2 hr, and diluted as required with saline containing 0·25% formalin. Each final suspension (1 ml containing 10^{10} cells) was emulsified with 1 ml of complete Freund's adjuvant and 1 ml of the mixture was injected intramuscularly into each hind leg of a rabbit. This was followed by two intravenous injections (0·5 ml containing 10^9 cells) 22 and 28 days after the first injection. The rabbits were bled 3 days after the final injection. Antisera against isolates NCPPB 450, NCPPB 451, and 0268a were prepared and stored frozen until required.

For agglutination tests, cell suspensions were made by harvesting the growth from slope cultures in saline containing 0·6% formalin and allowing these suspensions to stand overnight at room temperature. The agglutinin titres of the sera were determined after incubation of serum dilutions with a range of cell suspensions at 52°C for 4 hr.

For immunodiffusion tests, dense suspensions prepared as for agglutination were subjected to ultrasonic distintegration and the products used as antigens. Two-dimensional double diffusion in Petri dishes and on microscope slides was carried out with 1% Ionagar (Oxoid) containing 0·85% sodium chloride and 0·01% merthiolate.

(b) *Results*

On PYE agar after 3 days incubation at 28°C, the colonies of the vetch pathogen and of the authentic cultures of *Ps. stizolobii* were indistinguishable. In all cases discrete, well-separated colonies were 0·5–1·5 mm in diameter, circular, with an entire edge, amorphous, of low convex elevation, translucent to opaque, butyrous in consistency, and readily emulsified in water. After a further 4 days incubation the colonies had hardened and were difficult to remove from the surface of the agar. On PYE agar containing glucose the growth was more profuse and the colonies larger and more opaque. On this medium a similar change in consistency occurred, but more slowly and to a lesser degree. After incubation for 2–3 weeks the colonies became rubbery in consistency and adherent to the agar as described by Burkholder (1957).

No gross differences in morphology of the cultures of *Ps. stizolobii* and of the vetch pathogen were observed on PYE agar; all cultures were Gram-negative, straight rods with rounded ends, occurring singly or in pairs, the rods being less than 1 μm in width and 1·0–2·0 μm in length. In some preparations long thread-like forms were seen. After incubation for 48 hr on PYE agar containing glucose, Gram-stained preparations of cultures exhibited bipolar staining and contained refractile unstained inclusions. Preparations stained with Sudan Black B exhibited intensely sudanophilic cells. All cultures were actively motile in hanging-drop preparations.

Observations with the electron microscope have revealed the presence in all of the strains examined (0268a, 0296, 0300, NCPPB 450, and NCPPB 451) of a single

TABLE 3

COMBINED ADDITIONAL CHARACTERISTICS OF TWO ISOLATES OF *PSEUDOMONAS STIZOLOBII* FROM THE UNITED STATES OF AMERICA AND SIX ISOLATES OF A *PSEUDOMONAS* SPECIES FROM VETCH

All tests were carried out after incubation at 28°C unless otherwise indicated. + = positive, − = negative for the test concerned

Property	*Pseudomonas* sp. from Vetch	*Pseudomonas stizolobii*	Technique
Metabolism of glucose	Oxidative	Oxidative	Hugh and Leifson (1953)
Acid from carbohydrates:*			Hayward (1964)
Adonitol	+	+	
Arabinose	+	+	
Cellobiose	−	−	
Dulcitol	−	−	
Erythritol	−	−	
Fructose	+	+	
Galactose	+	+	
Glucose	+	+	
Glycerol	+	+	
Glycogen	−	−	
Inulin	−	−	
Lactose	+ (0300) − (others)	−	
Maltose	−	−	
Mannitol	+	+	
Mannose	+	+	
Melibiose	−	−	
Raffinose	−	−	
Rhamnose	+	+ (451) − (450)	
Salicin	−	−	
Sorbitol	+	+	
Sorbose	−	−	
Sucrose	−	−	
Trehalose	+	+ (451) − (450)	
Xylose	+	+	
Hydrolysis of:			
Aesculin	−	−	Hayward (1964)
Gelatin	−	−	Skerman (1967)
Starch	−	−	Skerman (1967)
Chitin	−	−	Skerman (1967)
Utilization of:			
Adipate	−	−	Stanier *et al.* (1966)
p-Hydroxybenzoate	+	+	Stanier *et al.* (1966)
Citrate (Simmons' medium)	+	+	Skerman (1967)
Citrate (Christensen's medium)	+	+	Skerman (1967)
Malonate	+	+	Skerman (1967)
Nitrate			
Nitrate to nitrite	−	−	Hayward (1964)
Gas from nitrate	−	−	Skerman (1967)
Nitrite destruction	−	−	Hayward (1964)

TABLE 3 (*Continued*)

Property	*Pseudomonas* sp. from Vetch	*Pseudomonas stizolobii*	Technique
Activity of:			
Arginine dihydrolase	−	−	Thornley (1960)
Arylsulphatase	−	−	†
Catalase	+	+	Skerman (1967)
Lipase (Tween 80)	−	−	Hayward (1964)
Lysine decarboxylase	−	−	Carlquist (1956)
Oxidase	−	−	Skerman (1967)
Phosphatase	+	+	†
Urease	+	+	Skerman (1967)
Liquefaction of pectate gel	−	−	Skerman (1967)
Gluconate oxidation	−	−	Hayward (1964)
Deamidation of acetamide	−	−	Buhlmann *et al.* (1961)
$CaCO_3$ from calcium lactate	+	+	Skerman (1967)
Green, diffusible pigment	−	−	Medium B of King *et al.* (1954)
Growth at:			
37°C	−	−	
15°C	+	+	
5°C	+	+	
Growth on:			
6% NaCl	−	−	‡
3% NaCl	+	+	‡
2% NaCl	+	+	‡
0·025% phenol	+	+	‡
0·1% phenol	−	−	‡
Growth at:			
pH 4·5	+	+	
pH 5·5	+	+	
pH 9·0	−	−	
Litmus milk:*			Skerman (1967)
Alkaline	+	+	
Reduction	+	+	
Peptonization	−	−	
Dihydroxyacetone from glycerol	−	−	Skerman (1967)
Phenylpyruvic acid from phenylalanine	−	−	Skerman (1967)
3-Ketolactoside from lactose	−	−	Bernaerts and de Ley (1963)

* Tubes incubated for 21 days.

† Tests for arylsulphatase and phosphatase activity were carried out by addition of filter-sterilized solutions of either the dipotassium salt of phenolphthalein disulphate or the disodium salt of phenolphthalein diphosphate to molten PYE agar medium to give a final concentration of 0·01%. After 7 days incubation slope cultures were flooded with 15% sodium carbonate solution; a red colour was indicative of a positive reaction.

‡ Tests for growth on sodium chloride and phenol media were made by addition of the respective chemicals to PYE liquid medium. Sodium hydroxide and hydrochloric acid were added to PYE liquid medium as required to obtain desired pH values.

polar flagellum of unusual thickness which has been shown to be sheathed (Fuerst and Hayward 1969). There are apparently no other records of sheathed flagella in the genus *Pseudomonas*.

The vetch isolates and the *Ps. stizolobii* strains were closely similar in antibiotic sensitivity pattern. All cultures were resistant to the following antibiotics: bacitracin, 5 units; ampicillin, 25 μg; colistin methane sulphonate, 200 μg; novobiocin, 5 μg; cloxacillin, 5 μg; and penicillin, 1·5 units. All cultures were sensitive to the following antibiotics: nalidixic acid, 30 μg; chloramphenicol, 10 μg; tetracycline, 10 μg; erythromycin, 10 μg; gentamicin, 10 μg; streptomycin, 10 units; nitrofurantoin, 100 μg; olenadomycin, 5 μg; neomycin, 10 μg. In the case of polymixin, 300 units, the results were variable; some cultures were sensitive to this antibiotic and others resistant or very slightly sensitive.

Other cultural and physiological properties are given in Table 3.

Agglutination reactions showed antigenic similarities among isolates NCPPB 450, NCPPB 451, 0300, and 0344a. Isolates 0268a and 0292 were antigenically similar but quite distinct from the other isolates (Table 4). Immunodiffusion tests confirmed the above results. Reactions of identity (fusion of precipitation lines) were noted with several antigens of NCPPB 450, NCPPB 451, 0300, and 0344a, whereas 0268a and 0292, although identical with each other, had no detectable antigenic relationship to the other group.

TABLE 4

TITRES OF AGGLUTINATION REACTIONS BETWEEN BACTERIAL SUSPENSIONS AND RABBIT ANTISERA, EXPRESSED AS RECIPROCALS OF ANTISERUM DILUTIONS

Antiserum	Bacterial Suspension					
	NCPPB 450	NCPPB 451	0300	0344a	0268a	0292
Anti-450	3200	3200	n.t.*	3200	<20	n.t.*
Anti-451	6400	6400	3200	6400	<20	<200
Anti-0268a	80	160	<200	200	6400	3200

* Not tested.

IV. DISCUSSION

The results show that the pathogen from vetch is *Ps. stizolobii*. The slight differences in host range observed between our results and those of Burkholder (1957) may reflect strain differences between the isolates, or differences in the techniques used for inoculation and incubation. *Trifolium subterraneum* and *Vicia faba* are new host records.

The cultural and physiological properties of *Ps. stizolobii* described in the paper are in close agreement with those of Burkholder (1957). Burkholder found that his isolates produced nitrite from nitrate in a broth medium but the reaction was stronger in a synthetic medium. In our work a semi-solid medium containing peptone (Hayward 1964) was used for the nitrate reduction test with a negative result.

For the rapid diagnosis of the pathogen, the features which taken together most clearly differentiate *Ps. stizolobii* from other plant pathogenic species of *Pseudomonas* are the following: presence of a single, polar, sheathed flagellum and of sudanophilic inclusions; negative reactions in the tests for oxidase, sucrose utilization, arginine dihydrolase, diffusible pigment production, gelatin liquefaction, and aesculin hydrolysis (Lelliott, Billing, and Hayward 1966).

The question arises as to whether a separate genus should be erected to contain this bacterial species. The possession of a single, sheathed, polar flagellum on a straight, rod-shaped bacterium with an oxidative metabolism of carbohydrates represents a unique combination of characteristics. However, such a step would be premature until other species of *Pseudomonas* have been examined for the presence of sheathed flagella.

At least some of the isolates are serologically similar to the isolates of *Ps. stizolobii* from the United States. However, some of the vetch isolates (e.g. strain 0268a) did not cross-react with antiserum prepared against NCPPB 450 and NCPPB 451. This result suggests antigenic heterogeneity among isolates of *Ps. stizolobii*.

Since heated suspensions of organisms were used to produce the antisera, only antibodies active against heat-stable antigens would have been formed. Thus, the serological similarities and differences noted between strains pertain to these antigens only. Furthermore, the cell surface antigens detected by agglutination are not necessarily the same as the soluble ones reacting in immunodiffusion.

The occurrence of *Ps. stizolobii* on white clover and velvet bean in areas where vetch had not been grown, and subsequent demonstration of the pathogenicity of these isolates to vetch, indicates that alternative hosts are a source of inoculum in the field. The occurrence of the organism on white clover and its importance to the vetch disease cycle deserves further investigation. The origin of *Ps. stizolobii* in Australia cannot be stated with accuracy. It is possible that it was introduced from the United States of America on velvet bean seed and became established on white clover at Grafton in 1938. During the last 10 years, however, large quantities of velvet bean seed have been imported from African countries where the pathogen is known to occur.

V. Acknowledgments

We gratefully acknowledge field assistance given by Mr. G. Kennedy and details of early records supplied by Mr. J. Walker.

VI. References

Bernaerts, M. J., and Ley, J. de (1963).—A biochemical test for crown gall bacteria. *Nature, Lond.* **197**, 406–7.

Buhlmann, X., Vischer, W. A., and Bruhin, H. (1961).—Identification of apyocyanogenic strains of *Pseudomonas aeruginosa*. *J. Bact.* **82**, 787–8.

Burkholder, W. H. (1957).—A bacterial disease of clover and velvet beans. *Phytopathology* **47**, 48–50.

Carlquist, P. R. (1956).—A biochemical test for separating paracolon groups. *J. Bact.* **71**, 339–41.

Dowson, W. J. (1957).—"Plant Diseases Due to Bacteria." 2nd Ed. (Cambridge Univ. Press.)

Fuerst, J. A., and Hayward, A. C. (1969).—The sheathed flagellum of *Pseudomonas stizolobii*. *J. gen. Microbiol.* **58**, 239–45.

HAYWARD, A. C. (1964).—Characteristics of *Pseudomonas solanacearum*. *J. appl. Bact.* **27** 265–77.

HUGH, R., and LEIFSON, E. (1953).—The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* **66**, 24–6.

HYNES, H. J., ET AL. (1941).—"Plant Diseases Recorded in New South Wales." Suppl. No. 2. [Bull. N.S.W. Dep. agric. Sci. No. 46.]

KING, E. O., WARD, M. K., and RANEY, D. E. (1954).—Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. clin. Med.* **44**, 301–7.

LELLIOTT, R. A., BILLING, E., and HAYWARD, A. C. (1966).—A determinative scheme for the fluorescent plant pathogenic pseudomonads. *J. appl. Bact.* **29**, 470–89.

ROTHWELL, A., and HAYWARD, A. C. (1964).—A bacterial disease of Bougainvillea. *Rhod. J. agric. Res.* **2**, 97–9.

SKERMAN, V. B. D. (1967).—"A Guide to the Identification of the Genera of Bacteria." 2nd Ed. (The Williams and Wilkins Co.: Baltimore.)

STANIER, R. Y., PALLERONI, N. J., and DOUDOROFF, M. (1966).—The aerobic pseudomonads: a taxonomic study. *J. gen. Microbiol.* **43**, 159–271.

THORNLEY, M. J. (1960).—The differentiation of *Pseudomonas* from other Gram-negative bacteria on the basis of arginine metabolism. *J. appl. Bact.* **23**, 37–52.

WOLF, F. A. (1920).—A bacterial leafspot of velvet bean. *Phytopathology* **10**, 73–80.