CHARACTERIZATION OF AN ISOLATE OF *PHYTOPHTHORA NICOTIANAE* VAR. *NICOTIANAE* FROM NORTH QUEENSLAND

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Abstract

An isolate of *P. nicotianae* var. *nicotianae* from North Queensland was shown to be of pathogenic race 0, since it was non-pathogenic to *Nicotiana longiflora* and *N. plumbaginifolia*, but was strongly pathogenic to *N. rustica* and *N. tabacum*. The pH optimum, the pH–growth response curve, the temperature optima for growth on various media, and growth in the presence of sterol were similar to those reported previously for isolates from America.

The utilization of sugars and amino acids by the Australian isolate was compared with that reported for isolates from other sources. The difficulties of evaluating such comparisons and the need for conducting such investigations under precisely defined conditions are discussed.

I. INTRODUCTION

Since its discovery in Java in 1896, *Phytophthora nicotianae* Breda de Haan var. *nicotianae*, the causal agent of black shank disease of tobacco, has been recorded in various countries of America, Africa, Europe, and Asia (Lucas 1965). The disease was first recorded in Australia in 1970, where it caused severe loss of tobacco crops in North Queensland (O'Brien 1970).

Black shank disease has been controlled for many years in the U.S.A. by the use of resistant tobacco varieties. Apple (1957) showed that natural populations of *P. nicotianae* were comprised of strains which varied in morphology, physiology, and virulence and later described two distinct pathogenic strains of the fungus, designated race 0 and race 1, which were separated by their virulence (race 1) or lack of pathogenicity (race 0) to *Nicotiana plumbaginifolia* Viv. (Apple 1962). Subsequently, Hendrix and Apple (1967) found that breeding lines developed from either *N. plumbaginifolia* or *N. longiflora* Cav. could be used as differential hosts to separate the two fungal races. Recently, Litton *et al.* (1970) screened the two races against 62 *Nicotiana* species and found that 48 were susceptible to both races, five were resistant to race 0, five were resistant to race 1, and four were resistant to both races.

The aim of the present study was to determine which race of *P. nicotianae* is present in Australia and to compare the cultural and physiological properties of the Australian isolate with those reported previously (Mehrota 1951; Wills 1954; Apple 1957; Roncadori 1965) for isolates from other countries.

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II. Experimental Details

(a) *Fungal Isolation and Identification*

In late 1970, a species of *Phytophthora* was isolated, using the lupin baiting technique of Pratt and Heather (1972), from the roots and surrounding soil of a diseased tobacco plant collected at Mareeba, Queensland, by Mr. W. Lovett. The species was identified as *P. nicotianae* var. *nicotianae* by the use of Waterhouse's (1963, 1970) keys and this was later confirmed by Dr. Stamps of the Commonwealth Mycological Institute, Kew (code number IMI 145495).

(b) *Pathogenicity Experiments*

Tobacco species and varieties were grown in 6-in. plastic pots filled with soil mix C supplemented with fertilizer IIc as described by Matkin and Chandler (1957) for 6–8 weeks under natural illumination at a day temperature of 22°C and a night temperature of 16°C. Mr. D. Wark, Division of Plant Industry, CSIRO, provided us with seed of *N. tabacum* L. cv. Virginia Gold, *N. tabacum* selection Coker 187, *Nicotiana* hybrid Dixie Bright 101, *N. plumbaginifolia*, *N. longiflora*, and *N. rustica* L.

Cultures were maintained on Difco lima bean agar. Zoospore production and inoculation methods were carried out according to Gooding and Lucas (1959). Inoculum was produced by blending mycelium grown in the medium of Hendrix *et al.* (1969). Whole plants were inoculated with blended mycelium (Apple 1957) and leaf strip tests using either blended mycelial or zoospore inocula were prepared by the method of Wills and Crews (1964). Fungal growth in stems was measured by the method of Hendrix and Apple (1967). Necrotrophy tests for leaf resistance were performed according to Shepherd and Mandryk (1967) with approximately 10^4 zoospores being applied to each leaf disk in the inoculum drop.

(c) *Growth Tests*

For comparison of growth on various carbohydrates, the casein hydrolysate basal medium of Shepherd and Pratt (1973) was used with the original carbon source replaced by an 8 mM concentration of the carbohydrate to be studied. Medium pH was adjusted to 6·5 before autoclaving at 10 lb/in.^{2} for 10 min, and 20 ml medium was dispensed into 125-ml conical flasks. The same basal medium, with sucrose as the carbon source, but with the casein hydrolysate replaced by other nitrogen sources, was used in studies of amino acid utilization. The original basal medium, with and without added sterol, was used in studies of the effect of β-sitosterol on growth. Following inoculation with a suspension of zoospores in water (approximately 10^5 zoospores per flask), flasks were incubated at 25°C. During the incubation period, each flask was shaken briefly by hand at 24-hr intervals. At the termination of the experiments, mycelium was harvested on a Millipore filter (porosity 1·2 μm), washed with 100 ml distilled water, and dried to constant weight at 95°C in tared weighing bottles. The mean dry weight of mycelium produced was calculated from three replicates in each experiment, experiments being repeated at least once.

Effects of temperature, presence or absence of sterol, and form of nitrogen source on mycelial growth rates were determined according to Shepherd and Pratt (1973). Electronically controlled incubators were used to maintain desired incubation temperatures to $\pm 0 \cdot 1$°C.

III. Results

(a) *Pathogenicity Tests*

Inoculation experiments were performed on 6-week-old seedlings of *N. tabacum* cv. Virginia Gold, *N. tabacum* selection Coker 187, *Nicotiana* hybrid Dixie Bright 101, *N. plumbaginifolia*, *N. longiflora*, and *N. rustica*, using either blended mycelial or zoospore inocula added to the supporting media. Plants were examined for disease symptoms 4 days after inoculation. Both inocula produced identical results and the pooled results of four such experiments were assessed.

In a second series of tests, the relative reactions of leaf disks obtained from plants of the above six hosts were rated following application of the necrotrophy test (Shepherd and Mandryk 1967).

A third series of tests were made to examine the extent of fungal growth in tobacco stems. The results of these three series of tests (Table 1) indicate that the North Queensland isolate of *P. nicotianae* var. *nicotianae* belongs to pathogenic race 0, when our data are compared with those of Litton *et al.* (1970).

TABLE 1

SUSCEPTIBILITY OF *NICOTIANA* SPECIES AND BREEDING LINES TO AN AUSTRALIAN ISOLATE OF *P. NICOTIANAE* VAR. *NICOTIANAE*

Host plant	Disease incidence of seedling plants*	Fungal growth in stems		Mean necrotrophy rating§
		Mean growth (cm)†	Plant status‡	
N. longiflora	0	0	8 healthy	0
N. plumbaginifolia	0	2·0	8 healthy	0·3
N. rustica	100·0	8·9	8 dead	4·8
N. tabacum cv. Virginia Gold	100·0	10·2	8 dead	5·0
N. tabacum selection Coker 187	66·6	7·0	5 dead, 3 healthy	1·9
Nicotiana hybrid Dixie Bright 101	20·8	5·7	1 dead, 7 healthy	2·1

* Disease incidence based on percentage of dead plants in four replicate experiments of six plants of each host.

† Growth of inoculum from point of inoculation, calculated as a mean from eight stems of each host.

‡ Plant status 8 days after stem inoculation.

§ Necrotrophy ratings were calculated by the method of Shepherd and Mandryk (1967) from replicates of 10 disks from each of six plants. Degree of reaction varies from 0 = no visible effects to 5 = multiple dark necrotic spots present.

Confirmation of this finding was given by a more limited series of tests, using the technique of Wills and Crews (1964). Strips were cut from leaves at mid-stalk positions, avoiding the leaf midvein, and one end of each strip was placed in contact with a blended mycelial inoculum of the fungus. After 5 days incubation at 25°C, the extent of necrosis was measured and the mean value calculated for 20 strips of leaves from four hosts. Necrosis extended for a mean distance of 42 mm in *N. tabacum* cv. Virginia Gold, 36 mm in *N. tabacum* selection Coker 187, 46 mm in *N. rustica*, and 18 mm in *N. longiflora*, this behaviour being consistent with the designation of pathogenic race 0 given previously.

(*b*) *Growth Experiments*

Separate lots of the sucrose–casein hydrolysate medium were adjusted to various pH values with hydrochloric acid or potassium hydroxide solution. The pH was measured at the start and termination of each experiment by means of a glass electrode pH-meter. Following incubation for 10 days at 25°C, dry weights of cultures that had been incubated at various pH levels in the range 3·0–8·7 were determined and the mean values of four replicate cultures at each pH were calculated.

The results (Table 2) indicate an optimum pH for growth within the range 6·0–7·5.

TABLE 2

EFFECT OF MEDIUM pH ON GROWTH OF *P. NICOTIANAE* VAR. *NICOTIANAE*

Initial pH	Final pH	Growth (mg dry wt.)*	Initial pH	Final pH	Growth (mg dry wt.)*
3·0	3·4	1·2	7·0	7·0	44·7
4·0	4·2	20·6	7·4	7·4	40·2
5·0	5·3	34·5	7·7	7·5	42·1
6·0	6·1	43·7	8·2	7·8	34·4
6·6	6·6	46·5	8·7	7·9	29·4

* Mean dry weight of mycelium (mg) produced after 10 days incubation at 25°C. Values represent the mean of four cultures at each pH level.

Growth rates (mm/24 hr) were determined on various media over the temperature range 15–39°C. Five replicate cultures were grown for each temperature–medium combination. The results (Table 3) are similar to those reported by other authors (Tucker 1931; Leonian 1934; Schwinn 1959; Waterhouse 1963) for isolates from other countries.

TABLE 3

EFFECTS OF INCUBATION TEMPERATURE ON RADIAL GROWTH RATES OF *P. NICOTIANAE* VAR. *NICOTIANAE* ON VARIOUS MEDIA

The values given are means of eight replicates

Growth medium	Radial growth rate (mm/24 hr) at:								
	15°C	20°C	25°C	27·5°C	30°C	32·5°C	35°C	37°C	39°C
Difco lima bean agar	0·6	0·90	1·45	1·60	*1·75**	1·65	1·10	0·15	0
V-8 agar	0·6	0·95	*1·10*	*1·10*	0·90	0·65	0·50	0·15	0
Oxoid malt agar	0·25	0·50	0·60	0·65	0·75	*0·80*	0·50	0·05	0
Oxoid corn meal agar	0·25	0·45	0·47	0·50	0·52	*0·55*	0·47	0·10	0
Casein hydrolysate–sucrose agar	0·40	0·50	1·00	1·05	1·15	*1·30*	0·70	0·10	0

* Optimum growth rates are printed in *italics*.

Shepherd and Pratt (1973) have reported that cultures of *P. drechsleri* Tucker were incapable of growth at 44°C, but when such cultures were returned to 25°C growth would sometimes recommence after a lag period. The tolerance to high temperature of the Queensland isolate of *P. nicotianae* var. *nicotianae* was compared with that of a number of other *Phytophthora* species, the results being shown in Table 4. Both *P. nicotianae* var. *nicotianae* and *P. nicotianae* var. *parasitica* (Dastur) Waterhouse showed similar tolerance to high temperature treatment and both showed a greater tolerance to such treatment than other *Phytophthora* species tested. Thus, while both varieties of *P. nicotianae* resumed growth immediately at 25°C following an 8- or 12-hr exposure to 44°C, *P. vignae* Purss recommenced growth only after a

1- or 2-day lag period respectively and all other species tested were apparently killed by such treatments.

TABLE 4

EFFECTS OF EXPOSURE AT 44°C ON SUBSEQUENT GROWTH AT 25°C ON CORN MEAL AGAR

Period of treatment at 44°C	2 hr					5 hr					8 hr				
Days of incubation at 25°C following high-temperature treatment	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Growth* at 25°C of:															
P. nicotianae, P. parasitica	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
P. vignae	+	+	+	+	+	+	+	+	+	+	0	+	+	+	+
P. citricola	+	+	+	+	+	0	+	+	+	+	0	0	0	0	0
P. drechsleri, P. megasperma	+	+	+	+	+	0	0	+	+	+	0	0	0	0	0
P. cinnamomi, P. cambivora	+	+	+	+	+	0	0	0	+	+	0	0	0	0	0
P. citrophthora, P. cryptogea, P. cactorum	+	+	+	+	+	0	0	0	0	+	0	0	0	0	0
P. syringae	0	0	+	+	+	0	0	0	0	0	0	0	0	0	0

* + = growth; 0 = no growth.

TABLE 5

UTILIZATION OF CARBOHYDRATES BY AN AUSTRALIAN ISOLATE OF *P. NICOTIANAE* VAR. *NICOTIANAE*

Sugar	Growth*	Final pH†	Sugar	Growth*	Final pH†
Glycerol	14·6	5·7	Raffinose	42·2	5·5
Xylose	9·9	5·9	Melezitose	42·0	6·3
Arabinose	3·9	6·2	Cellulose	1·5	6·4
Lyxose	2·0	6·4	Inulin	1·9	6·4
Ribose	2·0	6·4	Starch (soluble)	30·7	6·2
Rhamnose	2·3	6·4	Amylose	40·5	6·0
Fucose	3·8	6·3	Amylopectin	27·1	5·8
Mannose	11·0	6·4	Glucuronic acid	1·7	4·6
Galactose	12·2	6·1	Glucosamine	5·6	5·8
Glucose	20·5	5·7	*N*-Acetyl glucosamine	3·9	6·4
Fructose	25·1	5·8	Erythritol	1·7	6·4
Sorbose	2·7	6·1	Xylitol	1·4	6·4
Turanose	7·2	6·4	Sorbitol	2·1	6·4
Sucrose	42·7	5·4	Arabitol	2·1	6·4
Maltose	36·8	5·6	Mannitol	1·9	6·4
Cellobiose	4·1	6·4	Adonitol	2·5	6·3
α,α-Trehalose	2·5	6·4	Dulcitol	2·0	6·2
Lactose	2·8	6·4	No sugar	1·3	6·4
Melibiose	1·6	6·4			

* Growth = dry weight of mycelium (mg) produced after 7 days incubation at 25°C.
† Initial pH of the medium was adjusted to 6·5 before autoclaving.

(*c*) *Nutrition Experiments*

Mehrota (1951), Wills (1954), and Roncadori (1965) provide extensive data on the growth of *P. nicotianae* var. *nicotianae* on various carbohydrate sources. The

discrepancies between the accounts of these authors indicate, *inter alia*, the possibility of the occurrence of strains differing in their patterns of carbohydrate utilization. The present study, the results of which are shown in Table 5, was made to compare the behaviour of the Australian isolate with those reported by these authors.

The only detailed reports of utilization of different nitrogen sources by isolates of *P. nicotianae* var. *nicotianae* are those of Wills (1954, 1964). Table 6 shows similar results obtained with the Australian isolate, with various nitrogen sources added to give 0·32 g/l of nitrogen in the basal medium.

TABLE 6

EFFECT OF NITROGEN SOURCE ON RADIAL GROWTH RATE OF *P. NICOTIANAE* VAR. *NICOTIANAE*

Nitrogen source	Growth rate* (mm/24 hr at 25°C)	Final pH of medium	Nitrogen source	Growth rate* (mm/24 hr at 25°C)	Final pH of medium
Oxoid casein hydrolysate (acid)	16·5	5·6	Methionine	7·0	6·3
Asparagine	13·0	6·4	Glycine	6·0	6·2
Arginine	12·5	7·0	Cysteine	5·5	6·3
Glutamic acid	11·5	5·1	Potassium nitrate	4·0	6·3
Leucine	11·5	6·1	Phenylalanine	3·5	6·4
			Lysine	2·5	6·4

* Growth rates are the means of five replicates in the presence of 0·32 g/l of each nitrogen source.

Hendrix (1965) has shown that the growth of *P. nicotianae* var. *nicotianae* on glucose–peptone agar is increased in the presence of added cholesterol. A similar investigation by us, using sucrose–casein hydrolysate agar, showed that the mean growth rate of 10 replicate cultures was increased from 11·40 mm/24 hr in the absence of sterol to 16·50 mm/24 hr in the presence of 20 mg/l of β-sitosterol. The enhancement of growth of the Australian isolate in the presence of sterol is similar to that reported by Hendrix (1965) for American isolates of the fungus.

IV. DISCUSSION

When the results of the pathogenicity tests summarized in Table 1 are compared with the previous findings of Lautz (1957), Gooding and Lucas (1959), Wills and Crews (1964), Moore and Wills (1969), and Litton *et al.* (1970), the Australian isolate of *P. nicotianae* var. *nicotianae* is clearly of pathogenic race 0.

Further sampling of affected tobacco in North Queensland is necessary to determine whether the black shank organism present is only of race 0 or whether race 1 is also present. In any case, it would be desirable to use hybrids resistant to both races in any projected control programme, as Litton *et al.* (1970) have noted that cultivars resistant to race 0 in the U.S.A. allowed buildup and spread of race 1. The species *N. longiflora*, *N. repanda* Will., and *N. stocktonii* Brandeg. show resistance to both fungal races and resistant cultivars of *N. longiflora* × *N. tabacum* hybrids are commercially available.

Comparison of the cultural behaviour of the Australian isolate with that reported for isolates from the U.S.A. indicates similarities. Thus, the pH–growth response of the Australian isolate is similar to that reported by Apple (1957) for isolates from flue-cured tobacco in the U.S.A.

The effects of exposure to high temperatures on subsequent growth at 25°C (Table 4) have not been reported previously by other authors. Whether the technique will prove useful for species characterization will depend on the degree of variation to be found among separate isolates of the various species.

Hendrix (1965) showed the relative mean growth rates of two American isolates in the presence and absence of added sterol to be 1·238 and 1·437 respectively and these values are similar to that of 1·447 found in the present study.

Previous reports on the utilization of sugars by *P. nicotianae* var. *nicotianae* are difficult to evaluate because pH changed inconsistently during growth (Mehrota 1951), or because such effects have not been examined (Wills 1954; Roncadori 1965). However, there appear to be considerable differences in the behaviour of isolates from different sources. Thus, an Indian isolate showed substantial growth in the presence of sorbitol, mannitol, or dulcitol (Mehrota 1951), while one from the U.S.A. did not grow with these sugars as carbon sources (Wills 1954). It has also been observed that different isolates of various *Phytophthora* species, including *P. nicotianae* var. *nicotianae*, show different growth rates on various sugars (Roncadori 1965) and Roncadori considered that the utilization of a particular carbon source was a character of doubtful value for species separation. Nevertheless, notable differences do occur between our results and those previously reported. Both Mehrota (1951) and Wills (1954) reported that their isolates made negligible growth with raffinose as a carbon source, whereas we found this sugar to promote excellent growth. Mehrota (1951), Wills (1954), and ourselves have also shown that glycerol gives approximately the same growth rate as galactose, but Mehrota (1951) reported that erythritol, mannitol, dulcitol, and sorbitol support appreciable growth, whereas Wills (1954) and ourselves have found that sugar alcohols support only negligible growth. Clearly, further investigations conducted under defined conditions, particularly controlled pH, are necessary before such apparent differences can be evaluated.

A notable feature of the work of Mehrota (1951) and Wills (1954) was the long incubation periods used (30 days and 16 days respectively), whereas in the present study growth was found to be virtually complete after 7 days. We consider that the more rapid growth recorded by us is due to the addition of sterol in our experiments and that absence of sterol in those of Mehrota (1951) and Wills (1954) led to decreased growth rates.

In the investigation of Wills (1954) on amino acid utilization pH changes were not recorded, but in a later study (Wills 1964), in which various amino acids were added to a basal medium containing glutamic acid, the initial pH value of 6·5 was recorded as having fallen within the range 5·2–5·8 after growth had ceased. While direct comparison cannot be made, because of differences in the basal media used, our results support Wills' conclusions that glutamic acid supports good growth when used as a sole source of nitrogen and that no single amino acid was as effective in supporting growth as the complete mixture found in casein or its hydrolysate.

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