JASSA

Journal of Applied Science in Southern Africa
The Journal of the University of Zimbabwe

Volume 3, Numbers 1 and 2, 1997 **ISSN 1019–7788**

CONTENTS

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Vol. 3, Nos. 1 & 2, 1997
ISSN 1019–7788

CONTENTS

Characterisation of *Phytophthora nicotianae* isolates causing root and stem rot of tobacco[1]

A.J. Masuka[1] and C. Namichila[2]

[1]Kutsaga Research Station, P.O. Box 1909, Harare, Zimbabwe and [2] formerly Department of Biological Sciences, University of Zimbabwe,
[2]P.O. Box MP 167, Mt Pleasant, Harare, Zimbabwe.

Studies were undertaken to evaluate physiological and morphological variation, pathogenicity and races of 19 *P. nicotianae* isolates collected during the period 1985-1996 from infected tobacco in Zimbabwe. The growth of isolates was examined at a range of temperatures and potassium chloride-mediated osmotic potentials. Sporangia, oospore, oogonium and chlamydospore diameters were measured. Pathogenicity of isolates was determined on seedlings of *Nicotiana tabacum* and *Lycopersicorn esculentum* in greenhouse experiments. Optimum temperatures for growth of isolates were 25 °C and 30 °C. All isolates failed to grow at 37 °C. Growth of most isolates, based on a micro-well technique, was maximum at -0.14 and -1.00 MPa, and decreased at reduced water potentials. There were significant differences in the growth of an isolate at various temperatures and water potentials (p= 0.05). Length/breadth ratios of sporangia were 1.06:1 to 1.64:1. Chlamydospore, oogonia and oospore mean diameters were 22.5-27.5 µm, 22.5-32.5 µm and 20.0-24.0 µm, respectively. Although all isolates attacked tobacco and tomato, there were significant differences (p=0.05) in their pathogenicity. Significant differences (p = 0.05) in susceptibility of tomato cultivars to isolates were also observed. Based on the results of the tomato assay, all isolates were designated race 0.

Keywords: *Phytophthora nicotianae*, physiology, pathogenicity, tobacco, Zimbabwe.

Introduction

Phytophthora nicotianae Breda Haan, is a destructive and ubiquitous soil fungus in tropical and warm temperate climates attacking a wide range of plants including, citrus and solanaceous crops (Ferrin and Mitchell, 1985; Shew, 1991; Hall, 1993; Blaha *et al.*, 1994; Nielson, 1995). In Zimbabwe, *P. nicotianae* was first regarded as causing economic losses on tobacco in 1983 and by the 1990-93 growing seasons, stand losses of up to 30 percent were reported in irrigated tobacco (Tobacco Research Board, 1991). Losses have since been reported on dryland tobacco, and from most tobacco-growing areas of the country.

Three races of the fungus have been reported world-wide (Nielson, 1995), but two tested isolates from Zimbabwe have exhibited characteristics of race 0 (Larcout, 1995). The races have varying pathogenicity levels and host ranges (Larcout, 1995; Nielson, 1995), and this has important repercussions for fungus resistance breeding programmes. Races of *P. nicotianae* can be characterised by investigating reactions between and within *Nicotiana* species (Apple, 1962; Litton *et al.*, 1970; Nielson, 1995), determining the ability of isolates to produce elicitins *in vitro* (Pernollet *et al.*, 1992) and ascertaining their pathogenicity to *L. esculentum* plants (Larcout, 1995). Similar results have been obtained with these methods (Larcout, 1995). The morphology, physiology and pathogenicity of isolates have been used to characterise isolates of *P. nicotianae* (Oudemans and Coffey, 1991; Hall, 1993; Blaha *et al.*, 1994; Larcout, 1995). The optimum temperature for growth of *P. nicotianae* in culture is 28–32°C, but minimum and maximum temperatures are variable (Lucas, 1965; Hall, 1993). The availability of water to fungi is affected by matric and osmotic potentials, and although water

potential is a summation of the two, water potential on media will not differ whether it is altered by osmotic or matric means (Boddy, 1983). The desired water potential in media can be achieved by addition of solutes (Sommers *et al.*, 1970; Luard and Griffin, 1981; Boddy, 1983; Koske and Tessier, 1986).

The present study is part of a broader project on the biology, ecology, pathogenicity and control of *P. nicotianae* on tobacco and other solanaceous crops in Zimbabwe. In this paper we focus on the morphological and physiological variation and pathogenicity of isolates, and determination of races of the pathogen present in Zimbabwe.

Materials and methods

P. nicotianae isolates

Isolates of *P. nicotianae* were collected from roots and stems of tobacco plants showing typical fungus-induced symptoms (Shew, 1991) during 1985-1996 (see Table 4). Cultures were incubated at 25°C on oat meal agar (OMA) for 5-7 days, and morphological characters were used to confirm the identity of the fungus (Hall, 1993; Schmitthenner and Bhat, 1994). Isolates used in the study were taken from different, naturally-infected plants. Cultures were maintained on OMA, under mineral oil at room temperature (23-25°C).

Temperature studies

Mycelial disks (4 mm) from actively growing cultures were transferred to the edge of 9 cm Petri dishes. Two replicates for each isolate were incubated at 15, 20, 25, 30 and 37°C. Radial extension growth was measured along two lines at right angles to each other at the point of inoculation after four days. After all isolates tested failed to grow at 37°C, two replicates of isolates P14 and P25 were selected and incubated at 37°C for 3, 6, 9 and 12 days before being transferred to 25°C. The sub-cultures were examined daily for growth, for up to two weeks. The absence of growth at the end of this period was taken as evidence that the isolate had been killed at 37°C.

Examination of isolate morphology

Mycelial disks (4 mm) of isolates were sub-cultured centrally onto OMA in 9 cm Petri dishes and grown at 25°C under alternating light with 12 hours light/12 hours dark for seven days. Mycelium from the cultures was mounted in 60 percent cotton blue in lactophenol and the sizes of 20 each of chlamydospores, oogonia and oospores were measured. The lengths and breadths of 20 different sporangia were also determined.

Effect of water potential on fungal growth

Potassium chloride (KCL) was used to achieve the desired water potentials in the basal medium (BM). The composition of the BM was 7 g malt extract, 1 g Difco Bacto peptone, 0.5 g Difco Bacto yeast extract and 15 g agar in 1 litre distilled water. The amount of KCL required to obtain a particular water potential (MPa) was calculated from Koske and Tessier's (1986) formula:

$$g \text{ of KCL } L^{-1} \text{ of BM} = (\text{water potential} - 0.14) * 1.595.$$

The desired water potentials were -0.14 (un-amended medium), -1.00, -2.00, -3.00, -4.00 and -5.00 MPa. The procedure for autoclaving the medium is outlined in Masuka (1995), but micro-wells were used instead (Hoiland and Dybdahl, 1993). Ninety-six-well sterile polystyrene plates (Nunc-Immuno Plates) with 6.5 µm diameter wells were used. A column represented one water potential, but water potentials for each isolate were randomly allocated to columns 1-6 and 7-12 to avoid position effects. This method gives 8 replicates for each treatment per isolate. One hundred and fifty (150) µl liquid medium of the desired water potential was deposited in each well using a micro-pipette. Two isolates were used for each plate. Mycelial disks from actively growing cultures of isolates were cut and transferred to micro-wells. One column with un-amended medium (-0.14 MPa) was not inoculated. The plates were carefully covered in sterile plastic and incubated at 25°C for three days. Abundant growth had been observed previously in -0.14 and -1.00 MPa micro-wells after this incubation period. Two absorbance readings were taken at 490 nm by a spectrophotometer (Model MR 250) at the beginning and end of the experiment. The growth of an isolate at a given water

potential was regarded as the average absorbance differences in micro-wells of each treatment at the beginning and end of the experiment, adjusted for the control wells.

Growth of tobacco and tomato plants

Tobacco seedlings (*cv* Kutsaga Mammoth 10) were individually transplanted into 7 cm diameter pots with fumigated sandy loam soil five weeks after sowing. Tomato seedlings (*cv* Money Maker and Rodade) were transplanted into similar pots three weeks after sowing. The pots were kept in the greenhouse (22-30 C) and watered regularly.

Inoculum production

The method of Schmitthenner and Bhat (1994) was modified. Isolates were grown on OMA in Petri dishes for 7 days in darkness at 25°C. Mycelial tufts from cultures were scrapped aseptically and placed onto sterile pond water (Mg 0.99, Ca 2.60, Na 5.88, K 0.30, Cu 0.01, Mn 0.10, Zn 0.39, Fe 0.13, Cl 7.00, SO_4 3.70, CO_3 11.10 ppm) in Petri dishes. Tufts from one 9 cm Petri dish were sufficient for 10 ml pond water. The Petri dishes were incubated for 48 hours in pond water at 25°C, 50 cm below a continuous light source to induce sporangia formation. The Petri dishes were cooled for 30 minutes at -18°C to encourage zoospore release, and then left at 25°C for 60 minutes before three 1 ml-aliquots were taken for each isolate. The aliquots were shaken mechanically at 2000 rpm for 15 seconds to encsyt zoospores, before counting zoospores under the microscope with a haemocytometer. This method was found, from previous experiments, to enhance zoospore production by isolates. The zoospore and mycelial suspension of each isolate were aseptically blended for 1 minute. The inoculum concentrate was then ready for use.

Inoculation and disease assessment

The inoculum concentration for each isolate was adjusted to 1×10^4 zoospore/ml. Seventeen isolates of *P. nicotianae* were tested for pathogenicity on tobacco and tomato. The inoculation procedure for the two experiments entailed opening a hole 1 cm deep around the base of the seedling, pippetting 1 ml of zoospores and mycelial fragments into the hole, and immediately covering with soil. There were five replicates of each isolate randomised in five blocks for each experiment. Seedlings were kept in the greenhouse and watered regularly.

Seedlings were pulled after 7 days, washed and individually assessed for disease. A disease rating scale of 1-8 was used for the tomato experiment thus: 1 = no damage; 2 = < 1.0 percent damage to roots, stem with signs of discolouration upon sectioning, roots may/may not show discolouration; 3 = < 12.5 percent damage to roots, visible root/stem infection; 4 = < 25 percent damage to roots, stem blackening may be present, damage to tap root noticeable; 5 = < 50 percent damage to roots, stem blackening pronounced or absent, tap root affected; 6 = < 75 percent damage to roots, secondary roots have formed, tap root extensively dysfunctioned, wilting of leaves; 7 = < 100 percent severe damage to roots, secondary roots have formed, blackening of the stem, tap-root extensively dysfunctioned/missing, wilting of leaves and stem advanced and; 8 = dead. A shorter disease rating scale of 1-5 (1 = no disease; 2 = little stem and root infection, 3 = moderate infection, 4 = severe infection and 5 = dead) was used for the tobacco experiment, largely because of the low mortalities obtained with the tomato experiment.

Results

*Morphological variation among **P. nicotianae** isolates*

Sporangia lengths/breadths ratios ranged from 1.06:1 to 1.64:1, but 48.6 percent were between 1.20:1 and 1.40:1 (Figure 1). Most chlamydospores were found in the range 22.5–27.5 mm diameter, oogonia were predominately 26.0–30 mm, and the majority of oospores were 20.5 - 26.0 mm diameter (Figure 2). A summary of chlamydospore, oospore and oogonium data is presented in Table 1.

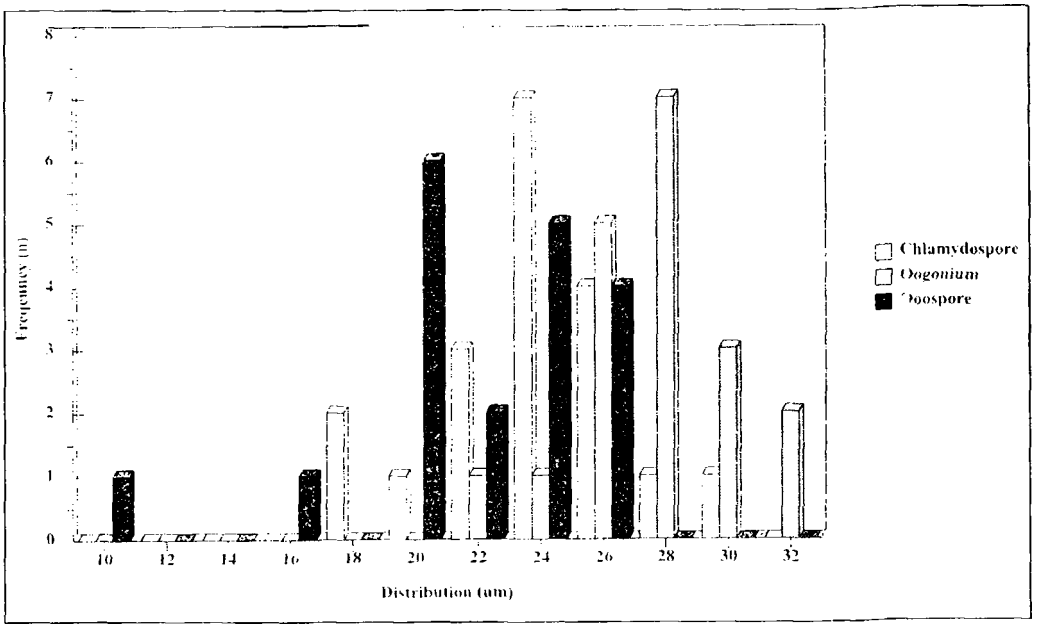

Figure 1: Length: breadth ratios of sporangia of *P. nicotianae* isolates.

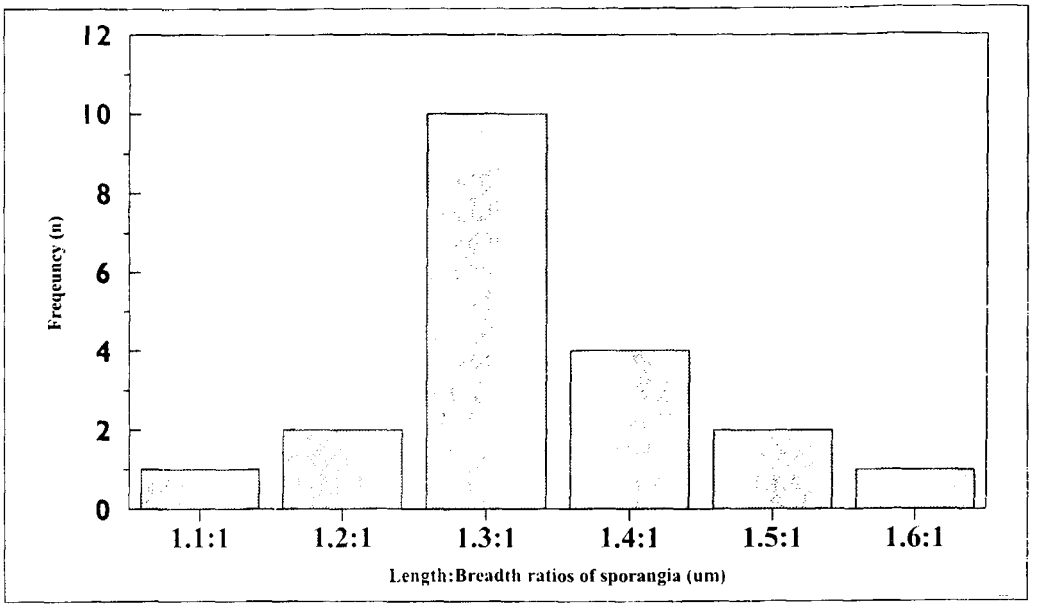

Figure 2: Distribution of chlamydospore, oogonia and oospore diameters of *P. nicotianae* isolates.

Table 1: Summary of morphological variation data in *P. nicotianae* isolates.

Parameter	Diameter (μm)		
	Chlamydospore	Oospore	Oogonium
Minimum	16.56	10.68	21.36
Maximum	28.53	25.97	32.14
Mean	22.70	20.95	26.85
SE	0.676	0.833	0.619

Effect of temperature on growth of P. nicotianae isolates

Growth rates were maximum at 25°C (8 isolates) and 30°C (15 isolates). None of the isolates tested grew at 37°C (Table 2). The effects of high temperatures (37°C) were more drastic than those of lower temperatures (15°C). The growth of an isolate at various temperatures was significantly different ($p = 0.01$), but there were no significant differences among isolates at a given temperature.

Table 2: Mean daily growth rate MM/day) of *P. nicotianae* isolates on Oat Meal Agar.

Isolate	15 °C	20 C	25 °C	30 °C	37 °C
P3	1.81	5.5	8.94	5.81	0
P12	3.25	5.94	8.81	9.31	0
P14	3.48	8	13.56	12.63	0
P15	2.63	7.63	12.69	12.63	0
P16	2.63	6.44	11.19	11.81	0
P18	2.19	5.94	10.38	6.44	0
P19	2.63	3.06	6.25	4.13	0
P20	4.25	6.5	11.81	11.75	0
P21	3.88	7	12.69	13.19	0
P23	2.81	7.13	11.94	12.06	0
P25	5.25	8.25	13.56	14.5	0
P26	4.56	8	13.56	13.63	0
P27	4.38	7.63	13.69	13.38	0
P28	3.5	7.25	10.81	11.38	0
P29	3.88	7.94	13.44	13.81	0
P30	6.5	8.63	13.56	13.31	0
P31	5.44	8.19	13.5	14.13	0
P32	4.5	7.69	13.38	14.31	0
P33	4.56	9.19	13.75	14	0

Isolate P14 grew at 25°C following incubation at 37°C for 3, 6, 9 and 12 days. Isolate P25 failed to grow only following incubation at 37°C for 12 days. The isolate was presumed to have been killed by prolonged incubation. Generally, longer incubation at 37°C resulted in prolonged recovery and growth at 25°C for both isolates.

Effect of water potential on growth of P. nicotianae

The majority of isolates grew best at -0.14 and -1.00 MPa (Table 3). There was a rapid decrease in growth at water potentials lower than those for optimal growth for each isolate. Isolates generally classified as being tolerant of reduced water potentials were p29, p20, p32 and p21; intermediate were p3, p27, p30, p33, p26, p19, p23, p12 and p31; while p14, p16, p15, p18, p25 and p28 were regarded as sensitive. These broad groupings were based on the overall performance of each isolate at various osmotic potentials. The growth of isolates was significantly affected by water potential ($p=0.001$), but did not vary significantly among isolates ($p=0.05$).

Table 3: Estimated growth of *P. nicotianae* isolates at various water potentials based on average light absorbance in micro-wells.

	Water Potential (MPa)						
Isolate	-0.14	-1	-2	-3	-4	-5	
P29	0.37±0.05	0.40±0.16	0.21±0.13	0.27±0.08	0.08±0.03	0.21±0.06	Tol.
P20	0.38±0.03	0.40±0.04	0.30±0.05	0.20±0.00	0.11±0.03	0.07±0.04	Tol.
P32	0.27±0.03	0.25±0.04	0.33±0.01	0.33±0.00	0.09±0.01	0.09±0.02	Tol.
P21	0.31±0.03	0.35±0.02	0.28±0.01	0.16±0.08	0.12±0.03	0.11±0.01	Tol.
P3	0.25±0.02	0.40±0.02	0.27±0.10	0.26±0.10	0.09±0.03	0.00±0.00	Int.
P27	0.34±0.09	0.11±0.06	0.32±0.05	0.24±0.03	0.09±0.01	0.00±0.00	Int.
P30	0.08±0.01	0.12±0.04	0.18±0.02	0.30±0.20	0.18±0.02	0.24±0.09	Int.
P33	0.20±0.04	0.20±0.00	0.13±0.02	0.19±0.02	0.11±0.01	0.24±0.02	Int.
P26	0.13±0.06	0.24±0.02	0.20±0.01	0.17±0.02	0.12±0.03	0.18±0.02	Int.
P19	0.24±0.01	0.18±0.03	0.24±0.02	0.11±0.01	0.18±0.06	0.08±0.07	Int.
P23	0.23±0.01	0.19±0.05	0.20±0.03	0.17±0.04	0.11±0.02	0.11±0.04	Int.
P12	0.24±0.04	0.23±0.00	0.13±0.02	0.17±0.02	0.15±0.01	0.08±0.04	Int.
P31	0.32±0.02	0.19±0.01	0.09±0.00	0.09±0.04	0.12±0.09	0.18±0.01	Int.
P14	0.20±0.04	0.27±0.00	0.16±0.01	0.10±0.03	0.10±0.05	0.04±0.03	Sen.
P16	0.36±0.06	0.21±0.03	0.22±0.01	0.03±0.04	0.01±0.02	0.00±0.00	Sen.
P15	0.32±0.00	0.29±.0.04	0.07±0.04	0.11±0.04	0.01±0.05	0.00±0.00	Sen.
P18	0.19±0.04	0.23±0.03	0.13±0.05	0.15±0.00	0.04±0.02	0.01±0.02	Sen.
P25	0.21±0.02	0.18±0.01	0.18±0.03	0.06±0.01	0.03±0.01	0.03±0.01	Sen.
P28	0.17±0.02	0.10±0.05	0.11±0.02	0.17±0.05	0.07±0.00	0.05±0.02	Sen.

The samples are grouped into tolerant (Tol.), intermediate (Int.) and sensitive (Sen.) categories.

Pathogenicity of isolates to tomato and tobacco seedlings

The most pathogenic isolate to tomato was p23 (mean score 7.2) on Rodade and the least was p27 (mean score 1.6) on Money Maker (Table 4). All isolates infected the two tomato cultivars, but Rodade was significantly ($p=0.05$) more susceptible. There were significant ($p=0.05$) differences in the infectivity of isolates on either cultivar. However, there was no significant interaction between isolates and cultivars. Isolates that infect tomato are generally categorised as race 0, while those that fail to attack it are race 1 (Larcout, 1995). On the basis of these results all isolates belong to race 0 of *P. nicotianae*. All isolates infected tobacco (Table 4) and differences in the pathogenicity of isolates were significant ($p=0.05$).

Discussion

The large morphological variations in sporangia lengths and breadths, and chlamydospore, oogonium and oospore diameters among isolates are consistent with previous studies (Waterhouse, 1963; Lucas, 1965; Oudemans and Coffey, 1991; Hall, 1993). The large morphological variation among isolates supports the view that these characters have little significance in the taxonomy of *P. nicotianae* at sub-specific level (Hall, 1993; Blaha *et al.*, 1994), contrary to earlier findings (Waterhouse, 1963; Lucas, 1965), and widespread use (Pernollet *et al.*, 1993; Csinos and Johnson, 1994; Larcout, 1995; Nielson, 1995; Sejalon-Delmas *et al.*, 1997).

The optimum temperature for growth of isolates in the range 25-30 °C is in agreement with studies elsewhere (Lucas, 1965; Hall, 1993). The optimum temperatures for growth of isolates are in the range optimal for tobacco growth during the cropping season in Zimbabwe. At 37°C, there was no growth probably due to temperature-induced interference with metabolic processes or a reduction in oxygen tension (Boddy, 1983). High temperature (37 °C) caused stasis, although prolonged exposure, as observed with isolate P25 after 12 days incubation, may cause death. However, the medium

Table 4: Pathogenicity of *P. nicotianae* isolates to tomato and toabcco seedlings.

Isolate	Location in Zimbabwe	Year isolated 19-	Lycopersicum *esculentum*		*Nicotiana tabacum*
			Mean Disease (scale 1–8)		Mean Disease (Scale 1–5)
			Money Maker	Rodade	
P3	Bindura	85	–	–	–
P12	Mvurwi	91	–	–	–
P14	Bindura	91	1.4	4	2.4
P15	Bindura	91	–	–	2.4
P16	Bindura	91	1.2	2	2.8
P18	Karoi	92	2.8	2.2	2.2
P19	Karoi	92	4.2	5.6	3.4
P20	Karoi	92	3.6	5.6	2.4
P21	Karoi	92	5.4	7	2.4
P23	Karoi	92	5	7.2	2.4
P25	Karoi	92	2.4	5.4	3.6
P26	Trelawney	94	2.4	4.6	4.6
P27	Karoi	94	2.4	1.6	3.4
P28	Banket	95	4	6.6	2.4
P29	Karoi	95	2.8	5.4	3.4
P30	Trelawney	95	1.26	3.6	2.4
P31	Karoi	95	4.8	7	3.6
P32	Banket	95	5.6	4.2	3.6
P33	Karoi	95	4.4	2.2	2.6
Mean			3.5	4.4	2.8
SED			1.39	1.48	0.60

*not tested

may influence survival at a given temperature (Hall, 1993).

The diameter of a colony is a satisfactory measurement of the rate of growth of a fungus because there is no acceleration of growth rate with time (Brancato and Golding, 1953). The gradual decrease in the colony radial growth is a reflection of the gradual temporal decrease in the rate of nutrient diffusion (Pirt, 1967).

Sommers *et al.*, (1970) found an optimal water potential for growth of - 1.00 MPa in studied *Phytophthora* species, using different media and growth assessment methods. The method reported here can therefore, be regarded as providing the likely response of *P. nicotianae* isolates to varying solute-mediated changes in water potential under laboratory conditions. As noted by Hoiland and Dybdahl (1993), the method enables rapid screening of many isolates, and is easy to set up, monitor, record and analyse results. Although KCL was the only osmoticant tested, previous studies (Sommers *et al.*, 1970; Boddy, 1983; Masuka, 1995) indicate that the general results obtained with this solute are representative of those obtained with other solutes. The general requirement for high water potentials for optimum growth of isolates is corroborated by field observations, where the fungus causes major losses in irrigated tobacco on the heavier soils. *P. nicotianae*, and other Oomycetes are generally, adapted to and thrive under moist conditions (Ferrin and Mitchell, 1985; Nielson, 1995; Shew, 1991; Hall, 1993).

The race characterisation studies indicated that only race 0 of *P. nicotianae* is present in Zimbabwe. The aggressiveness of *P. nicotianae* isolates is race-dependent (Larcourt, 1995). Isolates belonging to race 0 are less pathogenic than race 1 and race 2

isolates, and attack a wide range of solanaceous crops, including tobacco. Race 1 and race 2 isolates have also been found to be specific to tobacco, and more destructive. Furthermore, race 0 isolates produce elicitin, while race 1 isolates do not produce elicitin (Pernollet *et al.*, 1992; Larcourt, 1995). Elicitin is a major defence protein produced by some isolates which induces a hypersensitive reaction in challenged tobacco plants making them more resistant to infection by the pathogen (Sejalon-Delmas *et al.*, 1997). *P. nicotianae* is still a new fungus in Zimbabwe and breeding for resistance to the pathogen has only just begun. Perhaps as selective pressure is exerted on genotypes of the pathogen following the introduction of race-0 resistant cultivars there could be emergence of other, more pathogenic races of the pathogen. This situation will need to be monitored, both in the field and laboratory, to detect any changes in race composition early, and mitigate the potentially destructive losses that might occur in cultivars presumed to be resistant.

ACKNOWLEDGEMENT

We are grateful to Terttu Sigoboghla for technical assistance.

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