PHYTOPHTHORA NICOTIANAE ON TOBACCO AND ITS CONTROL IN SOUTH AFRICA

BY

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PREFACE

Phytophthora nicotianae, the causal agent of a disease known as black shank, has been a serious threat to tobacco cultivation worldwide. Black shank on tobacco has resulted in severe losses in quality and yield in most tobacco-producing areas of South Africa. Approximately 14000 ha of flue- and air- cured tobacco are planted annually in South Africa. The objective of this study was to consider various aspects of the biology and control of *P. nicotianae* in South Africa. Each chapter of this dissertation represents an individual entity prepared for publication over the course of a number of years. Therefore, some repetition between chapters has been unavoidable.

The introductory chapter presents a review of literature pertaining to all aspects of *P. nicotianae*, particularly its role as a pathogen on tobacco. The mechanisms of pathogenicity and control measures for the black shank disease are discussed. Particular emphasis is given to the origin of genetic diversity in *P. nicotianae* populations.

South African breeding programs aimed at obtaining tobacco cultivars with *P. nicotianae* resistance have been only partially successful. Even with the introduction of new resistant cultivars growers have reported an increase in black shank, which suggests that new races or more aggressive *P. nicotianae* populations may have emerged. In the second chapter of this dissertation, pathogenicity tests on tobacco cultivars were conducted. These tests were used to assess the variation in pathogenicity in the South African *P. nicotianae* populations. The results were also used to characterize isolates in terms of race. In addition, resistance in the commercially used South African tobacco cultivars to *P. nicotianae* races 0 and 1 was evaluated.

Metalaxyl is used worldwide for the control of *P. nicotianae* on tobacco. The continuous use of metalaxyl has, however, resulted in the development of metalaxyl resistance in *P. nicotianae* populations in Georgia (USA) and Korea. In South Africa the use of metalaxyl has recently given poor control of black shank in Potgietersrus, Rustenburg and Brits tobacco producing areas. However, no metalaxyl sensitivity studies have been conducted on *P. nicotianae* populations in South Africa. Chapter three reports on the screening of *P. nicotianae* populations for resistance against this compound. The results were used to determine whether the poor disease control could be attributed to decreased metalaxyl sensitivity. Field trials using metalaxyl with commercial cultivars were also conducted. The aim here was to determine if recently

developed cultivars with high levels of disease resistance, in combination with metalaxyl, give better disease control under field conditions.

The selection of cultivars and breeding lines for resistance to *P. nicotianae* has traditionally been conducted in fields where the pathogen is known to occur. Field trials are however; labour intensive, expensive and time consuming. The ability to screen large numbers of tobacco plants rapidly at the seedling stage is an essential component of effective black shank resistant breeding programs. Chapter four reports on the development of a rapid seedling-based screening technique to assay tobacco for resistance to *P. nicotianae*. This technique was compared with the stem inoculation technique commonly used on adult plants.

P. nicotianae is an important root and stem pathogen of many agricultural crops worldwide. Few genetic or population genetic studies have, however, been conducted on *P. nicotianae*. In chapter five RAPD's (Random Amplified Polymorphic DNA) were used to determine levels of phenotypic diversity within South African *P. nicotianae* populations, obtained from citrus and tobacco. These studies were undertaken to elucidate genetic differences between *P. nicotianae* populations from citrus and tobacco and to test for geographic differentiation. The identification of differentiation in *P. nicotianae* populations would assist breeders in selective breeding programs and planting regimes. Cultivars with resistance genes to specific *P. nicotianae* populations could then be cultivated in the appropriate tobacco regions.

All chapters of this dissertation relate to more effective and economical control of *P. nicotianae* on tobacco. Results of this study aim to contribute to a better understanding of the black shank disease in South Africa. Ultimately, breeding and deployment of resistant cultivars will provide the most sustainable and economical long-term approach to control *P. nicotianae* in commercial tobacco cultivation. This is best achieved against a background of a sound knowledge of the biology and genetics of the pathogen. It is my hope that the studies included in this dissertation will contribute, at least in part, to reducing the impact of this disease.

Chapter 1

Black shank caused by *Phytophthora nicotianae* on flue-cured tobacco in South Africa: A literature review

Black shank caused by *Phytophthora nicotianae* on flue-cured tobacco in South Africa

1. INTRODUCTION

The Oomycete genus *Phytophthora* includes more than sixty species (Stamps *et al.*, 1990; Erwin & Ribeiro, 1996). Most *Phytophthora* species are important pathogens of agricultural crops, natural ecosystems and forestry (Zentmyer, 1980; Erwin & Ribeiro, 1996). One of these species, *Phytophthora nicotianae* Breda de Haan is a soil borne pathogen of many different plant species (Ho, Ann & Chang, 1995; Erwin & Ribeiro, 1996; Ho & Lu, 1997). In South Africa, *P. nicotianae* is important both in forestry and agriculture causing severe root and stem diseases on Black Wattle (*Acacia mearnsii* De Wild.) (Margot, 1971; Zeijlemaker, 1971), *Eucalyptus* spp. (Belisario, 1990; Belisario, 1993), *Citrus* spp. (Le Roux *et al.*, 1991; Thompson, Phillips & Nel, 1995; Menge & Nemec, 1997) and tobacco (*Nicotiana tabacum* L.) (Lucas, 1975; Shew, 1991; Van Jaarsveld, 1995).

Approximately 14000 ha of flue- and air- cured tobacco are planted annually in South Africa. *P. nicotianae*, the casual agent of black shank, is responsible for severe losses in quality and yield in most tobacco-producing areas of South Africa (Prinsloo & de Villiers, 1977; Prinsloo, 1994; Van Jaarsveld, 1995) North America (Lucas, 1975; Stavely, 1979; Bickers, 1992), Australia (O'Brien, 1972; O'Brien & Davis, 1981), India (Gupta & Patel, 1978) and Java (Lucas, 1975). During the 1999 tobacco season in North Carolina, black shank was responsible for nearly half of all disease losses on flue-cured tobacco (Melton, 1999).

Van Breda De Haan first described *P. nicotianae* as the casual agent of black shank on tobacco during 1896 in Indonesia (Breda de Haan, 1896; Lucas, 1975). *P. nicotianae* infects both seedlings and older tobacco plants through the roots and stems. Symptoms include wilting, yellowing of leaves and the characteristic black lesion on the base or shank of the stalk (Lucas, 1975; Shew, 1991).

Controversy exists concerning the use of the names *P. nicotianae* and *Phytophthora parasitica* Dastur. Various authors use the name *P. parasitica* for the black shank pathogen (Tsao, 1971; Bonnet *et al.*, 1978; Förster & Coffey, 1990,1991). The name *P. parasitica* was originally used to describe the pathogen causing seedling blight of castor bean (*Ricinus communis* L.) (Dastur, 1913; Erwin & Ribeiro, 1996). Waterhouse (1963) found there were no morphological differences between the two species and thus *P. parasitica* was incorporated into *P. nicotianae* (Ho & Jong, 1989). DNA and isozyme comparisons (Panabières *et al.* 1989; Förster, Oudemans & Coffey, 1990b; Förster & Coffey, 1991; Oudemans & Coffey 1991; Hall, 1993) showed that the variants *P. nicotianae* and *P. parasitica* are not different. Many authors (Oudemans & Coffey, 1991; Brasier & Hansen, 1992) have proposed that the use of these variants should be discontinued and the proper taxonomic name *P. nicotianae*, be universally accepted (Erwin & Ribeiro, 1996).

P. nicotianae resides in the class Oomycetes, order Perenosporales and family Pythiaceae. *P. nicotianae* isolates are mostly heterothallic with two mating types, A1 and A2, but some isolates form limited numbers of oogonia and oospores in single spore cultures (Tsao *et al.*, 1980; Ko, 1981; Sansome, 1985). Oogonia are smooth and spherical with a diameter of 26.8µm. Antheridia are oval or spherical and amphigynous (Ho *et al.*, 1995). Interaction between hyphae of different mating type results in the formation of oospores (Ann & Ko, 1988; Chang & Ko, 1990). Sexual reproduction can result in increasing levels of genotypic diversity and may generate new combinations of virulence alleles (Romero & Erwin, 1969; Tooley, Sweigard, & Fry, 1986; Spielman, McMaster & Fry, 1989; Spielman *et al.*, 1990). As yet no population genetic studies have been undertaken on *P. nicotianae* populations. There is no evidence, which indicates the existence of the sexual cycle under field conditions.

Four races (races 0, 1, 2 and 3) of *P. nicotianae* have been described and are differentiated by host response (Apple, 1962; Prinsloo & Pauer, 1974; McIntyre & Taylor, 1978). Tobacco cultivar trials in South Africa have indicated the presence of race 0, 1, 2 and possible new races of *P. nicotianae* (Lamprecht, Prinsloo & Van Wyk, 1974; Prinsloo & Pauer, 1974; Nielsen, 1996).

Various genetic studies including mitochondrial DNA (mt DNA) restriction patterns (Hall, 1998; Lévesque, Harlton, & de Cock, 1998), species-specific DNA probes (Goodwin,

Kirkpatrick & Duniway, 1989) and RFLP's (Lacourt *et al.*, 1994) have been conducted on *P. nicotianae*. Lacourt *et al.* (1994) distinguished eight mt DNA haplotypes in 25 countries with one mtDNA haplotype being predominant with the broadest geographical distribution (Lacourt *et al.*, 1994). Colas *et al.* (1998) reported the first genetic evidence of a distinction between *P. nicotianae* isolates obtained from tobacco compared to other host plants using mitochondrial and nuclear DNA RFLPs.

The life cycle, mechanisms of pathogenesis and available control measures for *P. nicotianae* on tobacco will only be briefly discussed in this review since these topics have been treated thoroughly in a number of reviews (Lucas, 1975; Shew, 1991; Hall, 1993). Research on the taxonomy and pathogenicity of *P. nicotianae* on tobacco is summarized and the overall aims of the research described in this dissertation are presented.

2. LIFE CYCLE AND MECHANISM OF PATHOGENESIS IN *P. NICOTIANAE*

Oomycetes differ from the higher fungi in their biological characteristics (Zentmyer, 1983; Griffith, Davis & Grant, 1992). The Oomycete life history is primarily diploid (Sansome & Brasier, 1973) and cell wall components of *Phytophthora* spp. consist of cellulose and β-glucans rather than chitin which is typical of higher fungi (Bartnicki-Garcia, 1968).

Based on sequence analysis of the small sub-unit ribosomal DNA, Förster *et al.* (1990a) separated the Oomycetes from the Ascomycetes and Basidiomycetes. Oomycetes show a close evolutionary relationship to the Chrysophytes (Gunderson *et al.*, 1987; Förster *et al.*, 1990a; Bruns, White & Taylor, 1992). The phylum Oomycota has thus been placed in the Kingdom Chromista (Cavalier- Smith, 1986; Wainright *et al.*, 1993).

2.1 Asexual cycle

In *Phytophthora* spp., sporangia are produced in the asexual cycle. Sporangia of *P. nicotianae* are conspicuously papillate, broadly ovoid, obpyriform to obturbinate and usually non-caducous (Fig. 1) (Waterhouse, 1963). Sporangia can germinate directly through the formation of a germtube. This germtube can penetrate host tissue and is the first phase in the formation of vegetative hyphae. These vegetative hyphae can again

differentiate into new sporangia. Under specific environmental conditions, sporangia can also differentiate into zoospores (Fig. 2) (Bernhardt & Grogan, 1982; Ioannou & Grogan, 1984). Zoospores of *Phytophthora* spp. differentiate inside the sporangium and not in a vesicle outside the sporangium as are typical in *Pythium* species (Waterhouse, 1968; Robertson, 1980).

Zoospore release from sporangia is initiated under moist conditions such as after heavy rain and saturation of the soil profile. Zoospores have two flagella and are attracted to exudates from the roots of hostplants through chemotaxis. The ability of zoospores to actively move towards roots of their hostplant makes them formidable pathogens. In contact with the roots of the host zoospores encyst, germinate and form a germtube at the end of which appressorium-like bodies are formed that penetrate the host plant (Thompson & Allen, 1976; Bernhardt & Grogan, 1982; Shew, 1983).

In unfavorable environmental conditions such as decreasing soil moisture, chlamydospores may be produced from the vegetative hyphae (Tsao, 1971; Ioannou & Grogan, 1985). The spherical chlamydospores of *P. nicotianae* can germinate directly to form vegetative hyphae or sporangia or additional chlamydospores (Fig. 3) (Ioannou & Grogan, 1985; Hall, 1993; Ho *et al.*, 1995). Various authors have reported the survival of chlamydospores of *Phytophthora* spp. in soil for several years (Zentmyer & Mircetich, 1966; Weste & Vithanage, 1979; Sneh & Katz, 1988). Chlamydospores of *P. nicotianae* have been recorded to survive freezing in soil (Wills, 1964).

2.2 Sexual cycle

The sexual cycle of *P. nicotianae* involves two mating types, A1 and A2. Both mating types produce specialized mating structures (antheridia and oogonia) at a juncture on a suitable medium (Ko, 1981; Hall, 1993). In *P. nicotianae* the short antheridia have an amphigynous configuration with respect to the relatively small oogonia (Fig. 4) (Ko, 1981; Ho *et al.*, 1995). The oogonium grows through the antheridium and expands rapidly before the oogonial wall is penetrated. A single antheridial haploid nucleus is deposited in the oogonium to fuse with one of the oogonial haploid nuclei, forming a diploid nucleus. Meiosis occurs in the multinucleate gametangia (Shaw, 1983).

The single oospore that forms within the oogonium is globose and develops a thick inner wall (Waterhouse, 1963). The oospore wall consists of β-1,3-glucans (Lippman, Erwin & Bartnicki-Garcia, 1974; Bartnicki-Garcia & Wang, 1983) that enables the oospore to survive in diseased plant material (Slusher & Sinclair, 1973; Stack & Millar, 1985) and soil for long periods (Duncan & Cowan, 1980). Oospores of *P. nicotianae* are formed at temperatures lower (24 °C) than those required for optimal growth (28 – 32 °C) (Chern & Ko, 1994; Masuka & Namichila, 1996). Oospores germinate through formation of a germ tube, which gives rise to either vegetative growth or differentiates into sporangia with zoospores.

2.3 Mechanisms of pathogenesis

P. nicotianae causes a number of distinct symptoms on a tobacco plant. It can cause wilting of the seedlings, while symptoms on older plants include wilting and yellowing of leaves as well as characteristic black lesions at the base of stems (Figs. 5-6). When split, stems reveal longitudinally horizontal plate-like discs in the black pith (Fig. 7). In advanced stages of black shank, leaves turn brown, roots die, and plants are stunted (Fig. 8) (Lucas, 1975; Shew, 1991).

Leaf infections differ from root and stem infections. Soil containing the pathogen is splashed on basal leaves in periods of rainy wet weather. Symptoms on basal leaves include large circular lesions (8cm in diam.) that expand rapidly and become necrotic. *P. nicotianae* often grows from these lesions into the stem, causing more typical black shank symptoms (Lucas, 1975; Todd, 1981).

Recent studies on the mechanisms of pathogenesis in *Phytophthora* spp. include a better understanding of the homing response (host location) and infection by zoospores (Hardham *et al.*, 1994; Hardham, 1995). Homing responses in *Phytophthora* involves zoospore taxis, encystment, cyst adhesion, germination and germ-tube trophism (Zentmyer, 1961; Deacon & Donaldson, 1993).

P. nicotianae zoospores infect tobacco plants through the newly formed roots at the base of stems of young plants (Staub & Young, 1980; English & Mitchell, 1988c). Infection

is rapid since zoospores encyst and the cysts germinate within 30-40min (Deacon & Saxena, 1998).

3. CONTROL OF BLACK SHANK

In order to obtain effective disease control black shank integrated management programs are needed (Melton, 1998) as single components such as fungicide application and resistant breeding strategies have only turned out to be effective over the short term (Valleau, Stokes & Johnson, 1960). An integrated approach may contain the following components: (1) fungicide application, (2) phosphonate application, (3) cultural practices, (4) resistance breeding and (5) biological control. These are briefly discussed in the following sections.

3.1 Fungicide application

Various fungicides and soil fumigants have been used to control black shank (Taylor, Miller & McIntyre, 1975; Prinsloo, 1977; Kannwischer & Mitchell, 1978; Prinsloo, de Villiers & Norton, 1979; Reilly, 1980; Csinos & Minton, 1983). A black shank disease incidence of only 6% justifies application of the maximum fungicide rate for tobacco cultivation on economic grounds (Johnson, 1991). The fungicide metalaxyl (Ridomil G)® is presently widely used in South Africa for black shank control in tobacco cultivation (Van Jaarsveld, 1995). Metalaxyl (Ridomil G)® is applied in the ridge as a granule (22kg/ha) before planting with a follow up treatment 4 weeks later (11kg/ha) (Anonymous, 1999). Furthermore, metalaxyl (Apron 35 SD®) is also applied on tobacco seedlings as a drench in the seedbeds four weeks after seed germination. The Apron 35 SD® application is repeated in the seedbeds five days before planting as a preventive measure for infection of *P. nicotianae* (Anonymous, 1999).

Metalaxyl is a xylem-translocated phenylamide fungicide with an upward movement in plants (Edgington & Peterson, 1977; Cohen & Coffey, 1986). It prevents the formation of sporangia from chlamydospores and inhibits growth of mycelium in tobacco roots immediately after penetration (Staub & Young, 1980; Cohen & Coffey, 1986). Metalaxyl

also reduces the formation and germination of oospores of *Phytophthora infestans* (Mont.) de Bary (Hanson & Shattock, 1998).

Many authors have reported the appearance of isolates of *P. nicotianae*, less sensitive to metalaxyl after continuous use of metalaxyl in soil (Muino, Diaz & Jaenz, 1990; Csinos & Bertrand, 1994; Kim & Kang, 1997; Timmer, Graham & Zitko, 1998). Shew (1985) proposed that the periodic removal of the selection pressure by fungicide or crop rotation might minimize the selection of strains of *P. nicotianae* highly resistant to metalaxyl. Chang & Ko (1990) found that metalaxyl resistance segregates 3:1 for resistance being coded for as a single dominant gene in *P. nicotianae*. However, no genetic markers were used to confirm the identity of the obtained progeny. Inheritance of metalaxyl resistance has also been extensively studied in *P. infestans* (Shattock, 1988; Fabritius, Shattock & Judelson, 1997).

Metalaxyl was first introduced in South Africa during 1977 to 1978 as Ridomil SG® for control of *Phytophthora cinnamomi* Rands on avocado (*Persea americana* Mill.) (Darvas, 1983). Thereafter, Ridomil MZ® was used in Potgietersrus for black shank control on air-cured tobacco. Black shank was only effectively controlled for approximately two to three years in Potgietersrus before growers reported poor disease control (P. J. Smith, Lowveld Tobacco Growers Association, P. O. Box 60, Nelspruit, South Africa, pers. comm.) The use of grass (*Rhodes catambora* and *Cenchrus ciliaris*) crop rotation every second year has prolonged the use of metalaxyl (Ridomil G®) in flue-cured tobacco fields in the Lowveld and Groblersdal (Van der Linde, 1992; Prinsloo, 1994). In the Rustenburg and Brits area accelerated bio-degradation of metalaxyl in the soil is theorized to be the cause of poor disease control with metalaxyl (Ridomil Gold 2,5 GR®) (Anonymous, 1999). However, no metalaxyl sensitivity studies have been conducted on *P. nicotianae* populations in South Africa in order to establish if poor disease control is due to isolates less sensitive to phenylamides fungicides such as metalaxyl.

Chemical control alone will not end the occurrence of black shank in South Africa. This is because *P. nicotianae* survives in host tissue, and tobacco crops are annually or bi-annually planted (Van der Linde, 1992). Infection of previously uninfected fields occurs through irrigation and not all growers are financially able to use fungicides at the recommended rates.

3.2 Phosphonate application

Phosphonates (phosphonic acid) have been extensively used for control of *Phytophthora* spp. in Australia (Guest, Pegg & Whiley, 1995). Phosphonate produced by metabolism of the phosphonate ester provides protection to the plant and is the active ingredient of fosetyl-aluminium (Fosetyl-Al) (Fenn & Coffey, 1984). Fosetyl-Al is phloem-translocated with both downward and upward movement in the plant (Ouimette & Coffey, 1990).

Exposure to Fosetyl-Al stimulate defense responses in host plants such as hypersensitivity and increased phytoalexin accumulation (Guest, 1984; Guest *et al.*, 1995). The respiratory response seen in a compatible host-pathogen interaction is modified so that a pattern similar to that in an incompatible response is induced (Guest 1984; Guest, Upton & Rowan, 1989; Nemestothy & Guest, 1990). Formation of sporangia, chlamydospores and oospores of *P. nicotianae* are highly sensitive to Fosetyl-Al (Farih, Tsao & Menge, 1981; Coffey & Bower, 1984). Mycelial growth of *P. nicotianae* is also inhibited *in vitro* (Guest *et al.*, 1995).

Phosphonates are widely used as a spray on a wide range of crops. In South Africa a foliage spray of Fosetyl-Al (Alliette) is applied on tobacco approximately 6 weeks after planting (as soon as plants have sufficient leaves to absorb the chemical) with a follow up treatment 2 weeks later (Smith, 2000). Tree trunk injections are only used on tree crops such as avocado and citrus since the leaves take up the acid very slowly and do not transport it effectively to the roots where it is needed (Le Roux *et al.*, 1991; Guest *et al.*, 1995). The levels of phosphonate sensitives in populations of *P. nicotianae* in citrus orchard soils were not affected by trunk injections (Le Roux *et al.*, 1991). However, decrease in phosphonate sensitivity after prolonged use of phosphonate to control *Phytophthora* has been reported (Coffey & Bower, 1984).

3.3 Cultural practices

Cultural practices such as sanitation, nursery hygiene, soil drainage and regulated irrigation are important to control the spread of *Phytophthora* spp. in nurseries and fields (Collins &

Hawks, 1993). Furthermore, roots should be protected from nematode and insect damage since wounds provide entry sites for *P. nicotianae* (Shew, 1991).

3.3.1 Quarantine and nursery hygiene

Nursery hygiene and quarantine of tobacco seedlings are important since infected seedlings are a principal means by which the black shank pathogen can be spread (Tisdale & Kelley, 1926; Lucas, 1975; Shoemaker, Shew & Main, 1999). *P. nicotianae* first became a problem in tobacco fields in Australia in 1969, and the pathogen spread to farms via infected seedlings and aerial dispersal of sporangia (O'Brien & Davis, 1981). As quarantine measure, tobacco seedlings in South Africa are usually produced in a nursery on the farm where the seedlings are to be planted (Scholtz, Sonnenberg & Smith, 1998). The nursery area is fenced and has only one gate with a hand and footbath to prevent introduction of soil with pathogens. The water used for irrigation in seedbeds generally comes from fountains or rivers that are infected with *Phytophthora* spp. To deal with this problem, some growers chlorinate water prior to use (Von Broembsen, 1981).

Plants in commercial nurseries should be inspected for disease symptoms before they are distributed to growers. Seedlings can, however, be infected with *P. nicotianae* before obvious or typical symptoms of 'damping off' and 'black shank' appears (Lucas, 1975; O'Brien & Davis, 1981). The development of PCR-based detection techniques for *Phytophthora* spp. (Bonants *et al.*, 1997; Cooke & Duncan, 1997; Lacourt & Duncan, 1997) and other species-specific selective techniques (Benson, 1991; Unkles, Duncan & Kinghorn, 1992; Gabor *et al.*, 1993; Cahill & Hardman, 1994; Judelson & Messenger-Routh, 1996) could be useful for early detection of *P. nicotianae* in nurseries and fields. *P. nicotianae* in diseased tobacco samples from nursery and commercial fields have also been identified with another detection method based on enzyme-linked immunosorbent assays (Alert ™ *Phytophthora* Assay Elisa test kit) (Csinos & Miller, 1992).

3.3.2 Drainage

Effective drainage and control of irrigation water is essential for the control of *P. nicotianae* in seedbeds and commercial fields (Collins & Hawks, 1993). *P. nicotianae* is generally spread within fields by rainwater. This is from infested soil along rows, from infested fields to disease-free fields and into seedbeds. The pathogen is spread from the latter sources of inoculum into channels and rivers used for irrigation, causing new outbreaks in disease-free fields (Lucas, 1975; Shew, 1987; Shoemaker *et al.*, 1999).

Environmental factors such as drought followed by excessive rain are associated with severe black shank since soil saturation enhances infection through zoospore release and dispersal (Prinsloo, 1976; Shew, 1983; Ferrin & Mitchell, 1986b). Various authors found that low initial inoculum levels of *P. nicotianae* in soil can cause severe black shank since the inoculum of *P. nicotianae* multiplies continuously during the course of the epidemic (Nusbaum, Lucas & Chaplin, 1952; Kannwischer & Mitchell, 1978; Campbell & Powell, 1980).

Another factor determining the spread of *P. nicotianae* in the field is dependent on the level of host resistance (Shew, 1987). The spread of black shank in tobacco fields is slower on resistant than susceptible cultivars (Ferrin & Mitchell, 1986a; Ferrin & Mitchell, 1986b; Shew, 1987).

3.3.3 Crop rotation

Long-term crop rotations are widely recommended to reduce pathogen populations in soil but most tobacco growers find the latter uneconomical and have single year rotations (Lucas, 1975; Collins & Hawks, 1993; Csinos & Bertrand, 1994). Short crop rotation is ineffective for black shank control since *P. nicotianae* survives in the soil for 5 years and longer in the absence of tobacco (Lucas, 1975; Erwin & Ribeiro, 1996). Melton (1998) reported that, even if crop rotation and stalk and root destruction occurs, at the end of the tobacco season, black shank would not be controlled completely.

3.3.4 Host nutrition

Tobacco plants with nutritional imbalances such as excess levels of nitrogen (N), phosphates (P) and potassium (K) are more susceptible to black shank (Apple, 1961). By varying the levels of N, P and K on the tobacco variety Coker 139, it was shown that a high level of nitrogen increases disease levels (Apple, 1961; Melton, 1998) while different levels of potassium (K) and phosphates (P) had little effect. Phosphite, however applied as a root drench has been effective in protecting plants against invasion by *P. nicotianae* (Smillie, Grant & Guest, 1989). *P. nicotianae* is not killed by phosphite, but lesion extension is arrested in infected plants (Smillie *et al.*, 1989).

The development of black shank in the field is also related to soil Calcium and pH. Maximum disease occurs at high Calcium levels of 67% of cation-exhange capacity (Kincaid, Martin & Rhoads, 1970b). The recommended soil pH (pH6) for tobacco cultivation is ideal for black shank development (Kincaid *et al.*, 1970a). By lowering the soil pH (pH 5.8) Pearce & Nesmith (1997) found that the progression of black shank in the field was slower and pathogen concentration in the soil was lower the following season.

3.3.5 Nematode control

A synergistic disease complex has been reported between *P. nicotianae* and root-knot nematodes (Powell & Nusbaum, 1960). Root-knot nematodes not only cause wounds on tobacco roots but also induce the formation of giant cells. These giant cells whose physiology have been altered are easily invaded and colonized by the black shank pathogen (Lucas, 1975; Shew, 1991). An increase in nematode root damage was correlated with an increase in the incidence of black shank (Sasser, Lucas & Powers, 1955; O'Brien, 1972). It is, therefore, not surprising that nematicides have been reported to reduce the occurrence of black shank (Csinos & Minton, 1983; Csinos, Johnson & Golden, 1986; Csinos *et al.*, 1994).

3.4 Resistance breeding

The choice of tobacco cultivars generally determines the extent of disease loss, yield and quality of crops (Bowman *et al.*, 1984; Collins & Hawks, 1993). Breeding of tobacco cultivars with resistance to *P. nicotianae* is influenced by available genetic material from flue-cured and burley tobacco cultivars (Table 2), races of *P. nicotianae* present and environmental conditions.

3.4.1 Cultivars

Quality tobacco for cigarette production consists of a fine-tuned blend of yellow and orange tobacco. There are specific prerequisites for the end product of a breeding program. These are stipulated firstly by the cigarette manufacturers and secondly, by the growers. New cultivars must provide a balanced crop of yellow and orange tobacco with improved disease resistance, quality and yield (Bowman *et al.*, 1984). In 1992 there was an unforeseen overproduction of lemon yellow tobacco in South Africa. This resulted in many growers losing their farms because of decreased tobacco prices on lemon yellow tobacco (Scholtz, 1995).

The Lowveld Tobacco Growers Association and Agricultural Research Institute for Tobacco and Cotton are responsible for breeding tobacco cultivars with high quality, yield and disease resistance in South Africa (Fourie, 1992). Three cultivars (TL33, TL A4 and TL 9HON) have been traditionally planted in South Africa for 15 – 20 years. The tobacco cultivars TL A4 and TL 9HON are highly susceptible to black shank while TL33 has been evaluated as moderately resistant (Lamprecht & Prinsloo, 1977). Over a period of time, black shank has become increasingly more important on these commonly used cultivars and thus there is an urgent need for tobacco cultivars with improved disease resistance (Van Jaarsveld, 1995).

Breeding for black shank resistance has provided farmers with tobacco cultivars that now have high black shank resistance (90% of the plants are healthy) compared with previous cultivars (i.e. TL33) that were 60% resistant or TL A4 totally susceptible (Van Jaarsveld, 1995). The resistance base of these new cultivars is effective but may not be

durable. Apple (1967) reported the occurrence of race 1 of *P. nicotianae* after one year of planting cultivars with single gene complete resistance (Apple, 1962; Apple, 1967). Similar trends in the occurrence and spreading of race 1 have been reported in North Carolina (Litton, Collins & Legg, 1970) and Georgia USA (Csinos & Bertrand, 1994). This illustrates the need for more than single gene resistance, as well as, the importance of continuous resistance breeding to stay ahead of *P. nicotianae* populations able to overcome deployed resistance genes in the field.

Tobacco breeders have identified 5 main sources of resistance to *P. nicotianae* (Table 1). These are *Nicotianae longiflora* Cav., *Nicotiana plumbaginifolia* Viv., the flue-cured cultivar Coker 371-Gold and the cigar cultivars Florida 301 and Beinhart 1000-1 (*N. tabacum*) (Collins *et al.*, 1971a; Collins *et al.*, 1971c; Carlson *et al.*, 1997). Resistance genes from Florida 301 and Beinhart 1000-1 form the basis of resistance to *P. nicotianae* in most flue-cured and burley cultivars in the USA (Tisdale, 1922). Another source of resistance in burley tobacco originates from *N. longiflora*, which was used to breed the burley tobacco line L8 that has become the parent of 35% of burley acreage (Valleau *et al.*, 1960).

The origin of resistance in the flue-cured cultivar Coker 371-Gold is unknown (Csinos & Whitty, 1995) but some authors believe that the P*h* gene (*P. nicotianae* race 0 resistance gene) of Coker 371-Gold is derived from an interspecific source and closely resembles race 0 resistance in *N. longiflora* and *N. plumbaginifolia* (Campbell & Wernsman, 1994; Csinos & Bertrand, 1994; Carlson *et al.*, 1997). *N. plumbaginifolia* was used to breed the flue-cured variety NC2326 with resistance to race 0 of *P. nicotianae* (Table 2) (Chaplin, 1962; Chaplin, 1966).

Future breeding programs should aim to combine the P*h* gene (*P. nicotianae* race 0 resistance gene) in Coker 371-Gold with the high Florida 301 resistance for both races 0 and 1 of *P. nicotianae* (Carlson *et al.*, 1997). The use of double haploid technologies may also be a valuable tool to aid tobacco breeders in the development of desirable germplasm (Campbell & Wernsman, 1994).

3.4.2 Races of P. nicotianae

Four races (race 0, 1, 2 and 3) of *P. nicotianae* have been described and are differentiated by host response (Apple, 1957; Apple, 1962; Prinsloo & Pauer, 1974; McIntyre & Taylor, 1978), cold tolerance (Taylor, 1975; Taylor, McIntyre & Waggoner, 1978) and the presence of ketose (McIntyre & Hankin, 1977). Race 1 and race 0 are found in most tobacco-growing regions (Gupton, 1972; Lucas, 1975; Gupta & Patel, 1978; Nielsen, 1995). Race 0 was found in Zimbabwe (Masuka & Namichila, 1996) while races 1 and 2 have been reported in South Africa (Lamprecht *et al.*, 1974; Prinsloo & Pauer, 1974; Lamprecht & Prinsloo, 1977). Recent disease trials with the two cultivars, Coker 371 Gold and KY14 x L8 (with resistance to race 0) indicated the presence of race 0 in South Africa (Nielsen, 1995).

3.4.3 Environmental conditions

Climatic conditions (high moisture and temperature) most favorable for rapid growth and good quality tobacco is also conducive for sporangium formation and zoospore distribution of *P. nicotianae* (Gooding & Lucas, 1959a, 1959b; Dukes & Apple, 1968). High moisture and temperature increases disease development and severity of black shank in the field (Kincaid & Gratz, 1935; McCarter, 1967; O'Brien, Davis & Johnson 1981; Jacobi, Main & Powell, 1983; Ferrin & Mitchell, 1986b). Planting earlier when soil moisture and temperature are less favorable for the pathogen can reduce disease incidence of black shank (O'Brien & Davis, 1981). In addition to environmental factors influencing disease incidence, the level of resistance in the tobacco plant can also vary depending on environmental conditions (Crews, Wills & La Prade, 1964; Wills, 1964). Higher levels of aggression of the pathogen combined with lower levels of resistance in tobacco often result in high disease incidence (Crews *et al.*, 1964).

3.5 Biological control

Biological control agents can either control *Phytophthora* diseases or suppress growth of *Phytophthora* species (Daft & Tsao, 1983; Hoitink & Fahy, 1986; Chambers & Scott, 1995;

Berger *et al.*, 1996; Widmer, 1998). *Penicillium funiculosum* and the mycorrhizal fungi, *Glomus mosseae* and *Glomus intraradices* suppresses *P. nicotianae* by reducing the number of hyphae in the root cortex (Graham & Egel, 1988; Fang & Tsao, 1995; Cordier, Gianinazzi & Gianinazzi-Pearson, 1996; Trotta *et al.*, 1996). Other organisms antagonistic and mycoparasitic on *P. nicotianae* include *Chaetomium globosum* and *Trichoderma viride* (Heller & Theiler-Hedtrich, 1994).

The use of biological control agents in tobacco cultivation has not been extensively considered. English & Mitchell (1988a; 1988b) found that a composite of fungi and bacteria placed around tobacco roots inhibited *P. nicotianae*. This resulted in reduced plant mortality in soils infested with *P. nicotianae* (English & Mitchell, 1988b). Root rot on tobacco seedlings has also been effectively controlled with *Bacillus cereus* (Handelsman, Nesmith, & Raffel, 1991) and *Rhizoctonia* sp. (Cartwright & Spurr, 1998). Despite promising initial results under controlled conditions no biological control system is as yet effective under field conditions in South Africa.

4. GENETIC DIVERSITY OF *P. NICOTIANAE*

The ability to create and maintain genetic diversity in *P. nicotianae* populations will largely determine the durability of cultivar resistance. In highly diverse fungal populations host resistance and fungicide resistance may develop faster than in genetically uniform populations (McDonald & McDermott, 1993). Generating and maintaining genetic diversity in *P. nicotianae* is influenced by; (1) mutation, (2) sexual recombination, (3) heterokaryosis, (4) parasexuality (5) cytoplasmic inheritance, (6) migration, (7) center of origin, (8) genetic drift and (9) selection.

4.1 Mutation

Genetic diversity is created by mutations that alter heritable traits of an organism. The mutations may occur by base substitution, base deletion, base insertion and inversion-, duplication-, deletion or translocation of sections of DNA. These mutations occur at different rates and at random, with no constraints at the molecular level of DNA (Bos &

Stadler, 1996). Constraints are present at the physiological level where fitness of an individual may be altered. The occurrence of spontaneous mutations is rare and mutation rate is estimated between 10^{-3} to 10^{-6} for specific virulence loci (Zimmer, Schafer & Patterson, 1963). The effect of mutations in fungi is however, magnified by the large size of typical fungal populations and rapid sexual or asexual reproduction (Person, Groth & Mylyk, 1976). Asexual reproduction in fungi due to the formation of large numbers of single celled spores may increase the frequency of mutant alleles. Mutations in fungi may occur in any tissue since a distinct germline is lacking. These mutations are transferred sexually or asexually to the next generation.

Various authors (Apple, 1957; Colas *et al.*, 1998) have proposed that the black shank pathogen developed from rare mutational events in *P. nicotianae* followed by dissemination. Colas *et al.* (1998) suggested that the black shank isolates have evolved as a clonal lineage under genetic isolation, since low variability was found in the cluster of black shank isolates considered. Host specificity and the displacement of less virulent phenotypes is hypothesized to have resulted in the genetic isolation of *P. nicotianae* populations causing black shank (Lucas, 1975; Colas *et al.*, 1998).

Mutations have important implications for disease control of *Phytophthora* species since they alter the genetic make-up of the organism. Genetic changes conducive to poor disease control may include (1) the development of metalaxyl resistance (Davidse, 1981), (2) the development of tolerance to phosphonate (Bower & Coffey, 1985) (3) the appearance of new *P. nicotianae* races able to overcome cultivar resistance (Apple, 1957) and (4) the occurrence of more aggressive *P. nicotianae* isolates (Delon, 1991).

4.2 Sexual recombination

Phytophthora spp. are either homothallic or heterothallic in their sexual reproduction. *P. nicotianae* is a predominantly heterothallic species that requires both A1 and A2 mating types to initiate sexual reproduction and outbreeding (Johnson & Valleau, 1954; Förster & Coffey, 1990). Outbreeding among different *P. nicotianae* mating types should give rise to recombinant genotypes with high levels of heterozygosity (Goodwin, 1997). Förster & Coffey (1990) found evidence of sexual recombination in oospores of *P. nicotianae* where

oospore cultures had a high degree of variation in growth rate and colony morphology. However, no unambiguous evidence exists to suggest that *P. nicotianae* isolates outbreed either under laboratory conditions or field conditions.

The occurrence of homothallic isolates in *P. nicotianae* has been explained by the discovery of an extra chromosome belonging to the mating type complex (Sansome, 1985). Self-fertilization in the homothallic isolates of *P. nicotianae* will result in reducing the amount of heterozygosity by one half every generation. After seven generations of self-fertilization less than 1% of the original heterozygosity should remain (Goodwin, 1997). The role of sexual reproduction as it relates to population diversity in *P. nicotianae* in a natural environment is unknown (Förster & Coffey, 1990).

4.3 Heterokaryosis

Obtaining genetic evidence for the occurrence, and importance of heterokaryons in *Phytophthora* spp. is difficult (Brasier, 1992). In *Phytophthora megasperma* Drechsler and *P. infestans* pairings of mycelia or zoospores of different genotypes carrying different drug resistance and auxotrophic markers have implied heterokaryon formation (Leach & Rich, 1969; Long & Keen, 1977). However, no further evidence based on unequivocal genetic markers exists to demonstrate the occurrence of heterokaryosis.

4.4 Parasexuality

Parasexuality involves the transfer of genetic material from one organism to another without meiosis or the development of specialized sexual structures (Pontecorvo, 1956). Parasexual recombination provides a way by which the host range and pathogenicity of a fungus can be altered by somatic recombination of genotypes (Leach & Rich, 1969; Stephenson, Erwin & Leary, 1974). The frequencies of parasexuality in natural fungal populations and their importance are not known (Caten, 1981; Michelmore & Hulbert, 1987). In *Phytophthora* spp. the parasexual cycle would consist of (1) fusion of different diploid nuclei, (2) mitotic recombination, and (3) diploidisation through loss of the extra chromosomes. Heterokaryon formation (Long & Keen, 1977; Layton & Kuhn, 1988a), asexual karyogamy (Layton & Kuhn, 1988b) and hyphal anastomosis (Stephenson *et al.*, 1974) have all been reported

independently for *Phytophthora* species. However, no unequivocally data demonstrating the occurrence of a parasexual cycle exists.

4.5 Cytoplasmic inheritance

Cytoplasmic inheritance in the asexual cycle of *Phytophthora* spp. was first illustrated during 1968 when great phenotypic variation was found in zoospores of *P. infestans* (Caten & Jinks, 1968). Phenotypic characters such as rate of growth and sporangium production are carried from one generation to the next by cytoplasmic inheritance via zoospores (Caten & Jinks, 1968).

Cytoplasmic genomes in fungi consist of DNA plasmid genomes (Gunge, 1983), mitochondrial genomes (Tzagoloff, 1982; McNabb *et al.*, 1987) and double stranded RNA mycoviral genomes (Buck, 1980). Cytoplasmic genomes can be responsible for phenotypic variation through mutation, heteroplasmosis and copy number differences (Caten, 1987). These small genetic differences may lead to subtle continuous variation that may divide the population into discreet phenotypic classes (Fincham, Day & Radford, 1979). Double stranded RNA has been used to distinguish between different populations of *P. infestans* (Tooley, Hewings & Falkenstein 1989; Newhouse *et al.*, 1992).

Inheritance of mitochondrial DNA has been proposed to be uniparental in *P. nicotianae* (Förster & Coffey, 1990; Brasier, 1992). Whittaker, Assinder & Shaw (1994) proved with crosses of *P. infestans* that mitochondrial DNA is uniparentally inherited since they found no evidence of recombination or segregation. They could not, however, determine if mitochondrial inheritance is paternal or maternal. Fusion of *P. cinnamomi* zoospores under controlled laboratory conditions resulted in partial cytoplasmic exchange (Érsek & English, 1998). The importance of cytoplasmic inheritance in the creation of genetic diversity in *Phytophthora* populations warrants further investigation with cytoplasmic and genomic DNA markers.

4.6. Migration

Migration has been described as "a strong evolutionary force that has had a visible effect on the genetic structures of populations of *Phytophthora* species" (Goodwin, 1997). Migration and the replacement of old genotypes with new genotypes has been extensively studied in *P. infestans* (Spielman *et al.*, 1991; Fry *et al.*, 1992; Fry *et al.*, 1993; Goodwin *et al.*, 1994; Koh *et al.*, 1994), *P. cinnamomi* (Linde *et al.*, 1997) and *Phytophthora sojae* Kaufmann & Gerdemann (Drenth *et al.*, 1996). Little information is available on migrations of *P. nicotianae*. Genetic studies on isolates of *P. nicotianae* from the United States, Puerto Rico, Colombian, Cuban, Greek and Bulgarian indicate two separate long distance migrations from Central America to north America and to the Balkans (Colas *et al.*, 1998). There is no information concerning the origin of black shank in other parts of the world such as Australia and South Africa.

4.7 Center of origin

Very little is known regarding the origin of *P. nicotianae* causing black shank on tobacco. Colas *et al.* (1998) suggested that the black shank epidemic in the United States originated in southern Georgia during 1915. Thereafter, the black shank epidemic invaded Florida (Tisdale, 1922; Tisdale & Kelley, 1926) and spread to Tennessee and Kentucky for the following 20 years. The discovery of black shank in Puerto Rico in 1924 (Lucas, 1975) led Colas *et al.* (1998) to suggest that the pattern reflects the diffusion of a single strain, that might have originated in Central America. This theory is supported by the results of Colas *et al.* (1998) where Cuban and Columbia isolates of *P. nicotianae* occurred in the same cluster. This cluster also included some Greek and Bulgarian cultures, which are probably the consequence, of a long-distance transportation event from North America to the Balkans where the disease was not recorded until 1930. Colas *et al.* (1998) also found another cluster consisting of highly virulent isolates from the United States and Transvaal (South Africa). They proposed that this cluster had a different origin since isolates in this group had different mitochondrial haplotypes and lack the ability to produce elicitin. The center of origin of only a few *Phytophthora* species has been unequivocally established through

extensive population studies for example *P. infestans* and *Phytophthora mirabilis* Galindo & Hohl in Central Mexico (Goodwin & Drenth, 1997). However, for most of the *Phytophthora* species data on the original host plants as well as diversity on a global scale is lacking to unequivocally demonstrate centers of origin for the remaining species.

4.8 Genetic drift

The migration of *Phytophthora* populations to previously unoccupied territories results in the establishment of founder populations. Founder populations contain a small fraction of the overall genetic diversity, therefore, genetic drift may occur during the founder event and in subsequent generations (Nei, Maruyama & Chakraborty, 1975; Maruyama & Fuerst, 1985). Genetic drift in *Phytophthora* populations is the result of founder effects and reductions in population size (Goodwin, 1997). Population size in most plant pathogens is reduced between seasons with sanitation practices, fungicide use, the absence of hosts and the lack of oospores (Fry *et al.*, 1992). Genetic drift becomes increasingly important with decrease in population size and will be more severe if only one mating type of a heterothallic *Phytophthora* species survives (McDermott & McDonald, 1993; Goodwin, 1997). Genetic drift "can lead to different allele frequencies or the fixation of different alleles in isolated populations through random sampling of genes over generations" (McDermott & McDonald, 1993). Genetic drift has been described as the most probable explanation for the population genetic structure in *P. infestans* populations outside Mexico (Fry *et al.*, 1992).

4.9 Selection

Hosts provide a major selective influence in *Phytophthora* evolution, where selection occurs for pathogenicity genes and specific virulence genes (Brasier & Hansen, 1992; Goodwin, 1997). The cultivation of resistant tobacco cultivars has clearly resulted in the selection of races of *P. nicotianae* able to overcome cultivar resistance (Delon, 1991; Melton, 1998). Similar host pressure caused the selection of new races and a rapid turnover of genotypes within populations of *P. infestans* (Fry *et al.*, 1991; Fry *et al.*, 1992; Miller, Johnson &

Hamm, 1997) and *P. sojae* (Ryley *et al.*, 1998). The use of fungicides in agricultural practice has resulted in the appearance of fungicide resistance genes in *P. nicotianae* (Shew, 1985; Chang & Ko, 1990) and *P. infestans* (Koh *et al.*, 1994; Goodwin, Sujkowski & Fry, 1996).

5. CONCLUSIONS

- The 30 million-kg tobacco crop, cultivated annually in South Africa is threatened by the presence of black shank. The occurrence of black shank has grown from sporadic reports (Du Pisanie, 1990) to one of the most devastating and widely found tobacco diseases in the country (Prinsloo, 1994; Van Jaarsveld, 1995).
- The use of cultural practices, resistant cultivars and chemicals has been partially successful in controlling black shank. Unfortunately, growers have reported the disease on cultivars previously described as resistant to race 0 of *P. nicotianae*.
- Preliminary trials indicate the presence of isolates of *P. nicotianae* resistant to metalaxyl that is used for black shank control.
- The presence of *P. nicotianae* in rivers used for irrigation, as well as the practice of distributing seedlings from one geographical area to another, creates ample opportunity for dissemination of metalaxyl resistant and virulent strains of the pathogen.
- Very little is known regarding mating types, genetic diversity, and variation in virulence of *P. nicotianae* isolates in tobacco fields in South Africa. Furthermore information is lacking on the pathogenicity of citrus isolates of *P. nicotianae* towards tobacco and vice versa. Old citrus orchards are presently converted to tobacco fields and these isolates may form a source of inoculum.

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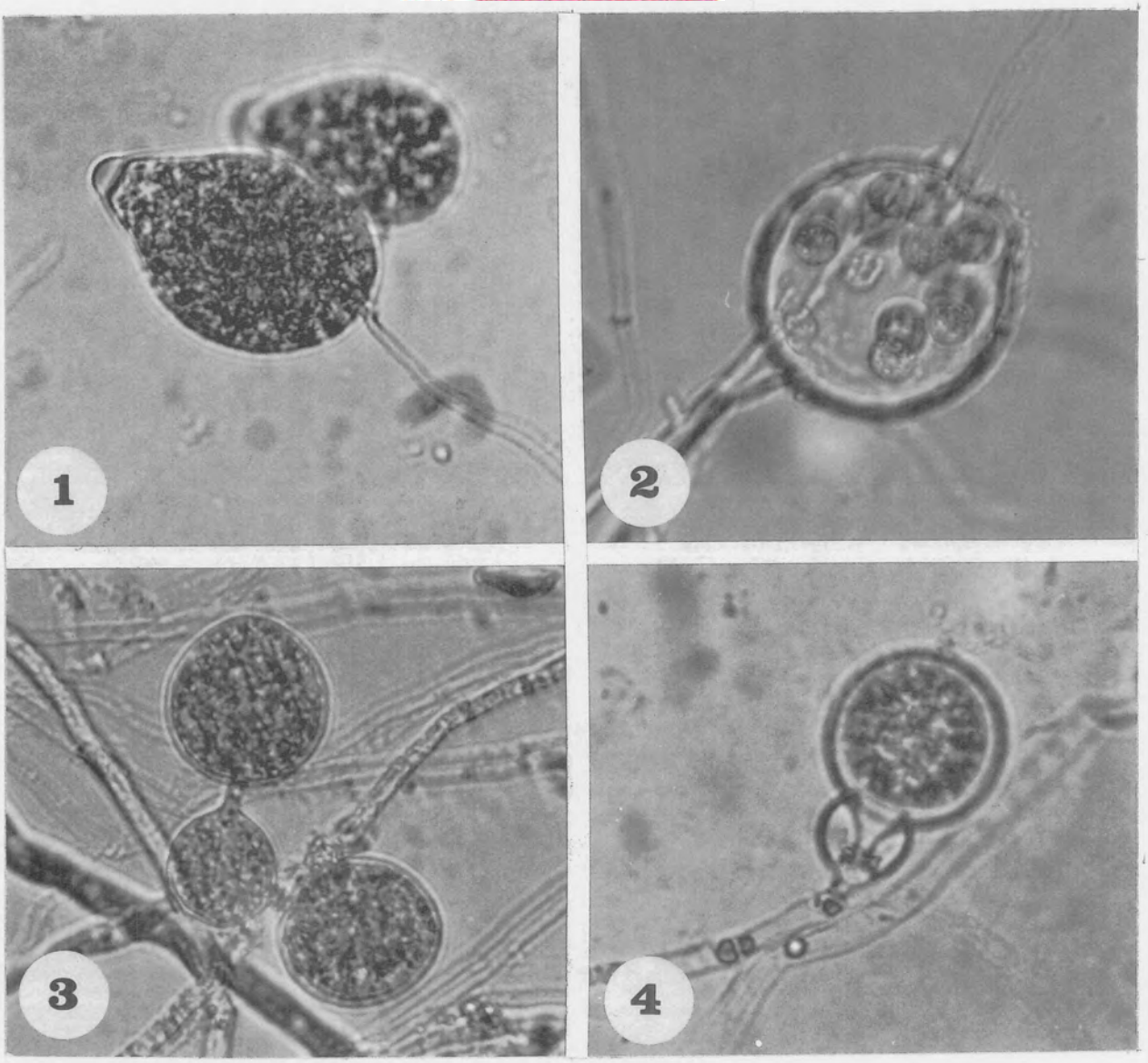

Figs. 1-4 Morphology of *Phytophthora nicotianae*. **Figs. 1-2** Papillate sporangia with zoospores of *P. nicotianae*. **Fig. 3** Chlamydospores of *P. nicotianae*. **Fig. 4** Antheridia of *P. nicotianae* have an amphigynous configuration with respect to the oogonia.

Figs. 5-8. Tobacco plants infected with *Phytophthora nicotianae* exhibiting black shank symptoms. **Figs. 5-6** Older plants exhibiting symptoms of wilting, yellowing of leaves and black lesions (arrow) at the base of stems. **Fig. 7** Plants infected with *P. nicotianae* reveal stems with longitudinally horizontal plate-like discs (arrow). **Fig. 8** Severely stunted tobacco plants in an advanced stage of black shank (arrow).

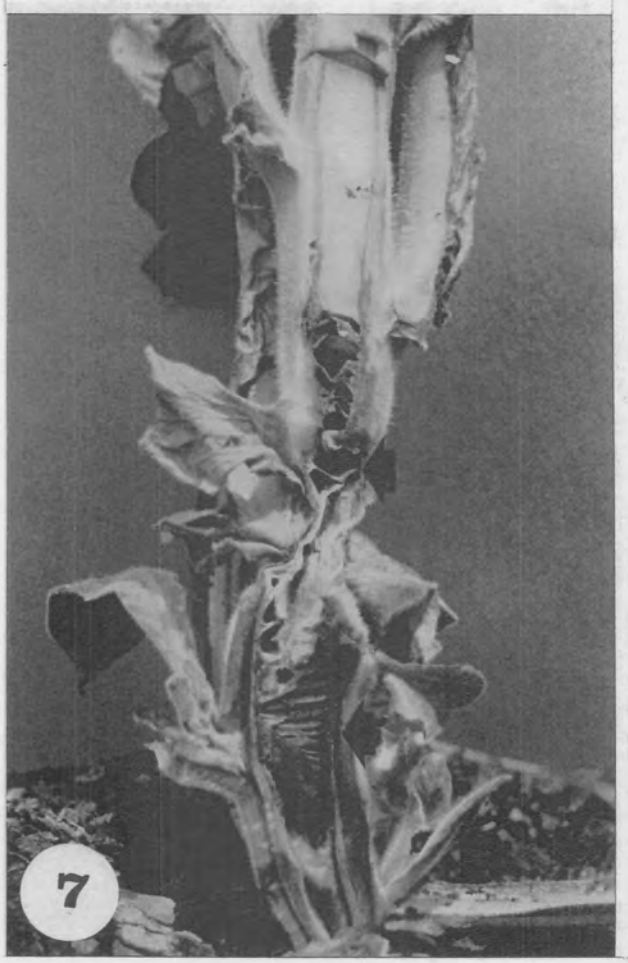

Table 1. Sources of resistance to *P. nicotianae*

Sources of resistance	Type of resistance	Reference
Nicotianae tabacum Florida 301	Polygenic partial resistance to race 0 and race 1	Tisdale (1931) Apple (1967) Wernsman, Matzinger & Powell (1974) Lucas (1975)
Nicotianae tabacum Beinhart 1000-1	Oligogenic partially dominant resistance to race 1	Silber & Heggestad, (1963) Chaplin (1966)
Nicotianae longiflora	Single dominant gene with resistance for race 0	Valleu *et al.*, (1960)
Nicotianae plumbaginifolia	Single dominant gene with resistance for race 0	Chaplin (1962)
Coker 371-Gold	Single dominant P*h* gene with complete resistance to race 0 of *P. nicotianae*	Carlson *et al.* (1997)

Table 2. Tobacco cultivars used in breeding trials for *P. nicotianae* resistance

Cultivars	Resistance	Reference
KY 14 (Burley)	Most susceptible, non-resistant variety	Collins *et al.* (1971b) Tedford & Nielsen (1990) Nielsen (1991, 1995)
KY 14 x L8 (Burley) L8 resistance from *Nicotianae plumbaginifolia*	L8 specific resistant (R1 gene) to race 0 Susceptible to race 1	Litton *et al.* (1970) Nielsen (1991, 1995)
Burley 37	Moderately resistant to race 0 and race 1	Collins *et al.* (1971b) Gupton (1972) Nielsen (1991)
Coker 371 Gold (Flue-cured)	Highly resistant to race 0, Susceptible to race 1	Nielsen (1991) Cartwright, Spurr & Sisson (1995) Carlson *et al.* (1997)
NC 2326 (Flue-cured)	Low resistance to race 0 Susceptible to race 1	Chaplin (1962, 1966) Litton *et al.*, (1970) Nielsen (1991, 1996) Cartwright *et al.* (1995) Wernsman *et al.* (1974) Csinos (1999)

Table 2. (continue)

Cultivars	Resistance	Reference
Hicks	Susceptible to race 0	Csinos (1999)
	Susceptible to race 2	Lamprecht *et al.* (1974)
Beinhart 1000-1	Non-specific very high resistance to race 0, race 1 and race 3.	Silber & Heggestad (1963)
		Chaplin (1966)
		Litton *et al.*., (1970)
	Resistant to race 2 has never been tested.	Stavely (1979)
		Tedford & Nielsen (1990)
		Nielsen (1996)
NC 1071	Immune to race 0	Litton *et al.*, (1970)
Resistance from *Nicotianae longiflora*	Resistant to race 3	McIntyre & Taylor (1978)
	Susceptible to race 1	Stavely (1979)
TL33	Resistance to race 1 and 2	Lamprecht & Prinsloo (1977)
		Nielsen (1994, 1997)

Chapter 2

Evaluation of tobacco cultivars for resistance to races of *Phytophthora nicotianae* in South Africa

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Evaluation of tobacco cultivars for resistance to races of *Phytophthora nicotianae* in South Africa

Black shank caused by *Phytophthora nicotianae* is a destructive root and stem disease of cultivated tobacco. *P. nicotianae* isolates associated with black shank in South Africa were selected in order to evaluate their aggressiveness, designate races of *P. nicotianae* and to determine which tobacco cultivars are resistant to them. Stem inoculations were conducted in the greenhouse using 32 *P. nicotianae* isolates from different tobacco growing regions in South Africa. The 32 *P. nicotianae* isolates differed significantly in levels of aggressiveness. Race 0 and 1 of *P. nicotianae* occurred in most of the tobacco growing regions. Eight isolates were selected on the basis of geographical origin and virulence for race characterization using a set of differential tobacco cultivars. South African race 0 and race 1 isolates were used to evaluate black shank resistance of 11 commercially planted tobacco cultivars. Commercially planted cultivars differed significantly in their resistance to race 0 and 1. Cultivars Vuma/3/46 and LK3/46 were highly resistant to both race 0 and 1 while cultivars LK33/60 and OD1 were highly resistant to race 0 but susceptible to race 1. Results of this study provide valuable information on cultivar resistance and selection of *P. nicotianae* isolates for future breeding programs in South Africa.

Black shank, caused by *Phytophthora nicotianae* Breda de Haan is the most important cause of tobacco quality and yield loss in the United States (Bickers, 1992; Melton, 1998) and South Africa (Prinsloo, 1994; Van Jaarsveld, 1995). Plants infected early in the season are severely stunted and collapse before leaves are sufficiently mature to be harvested (Csinos & Bertrand, 1994). Disease symptoms vary with age of plants and include wilting, leaf yellowing and tissue degeneration at the base of blackened stems (Lucas, 1975; Shew, 1991).

In South Africa, tobacco is cultivated in three main areas. These include Mpumalanga (Lowveld Golden Leaf Ltd. in the Lowveld trading area), North-West Province (MKTV Co-operative in the MKTV trading area) and Northern Province (Potgietersrus Co-operative in the PTK trading area). Approximately 25 million kg flue-cured and 4.8 million kg air-cured tobacco is produced annually in these regions (Anonymous, 1998). The gross income from agricultural production of tobacco in South Africa amounts to 423 million Rand (J. S. Venter, Tobacco Board RSA, P. O. Box 26100, Arcardia, 0007, pers comm.). However, the occurrence of black shank in most of the South African tobacco growing regions poses a serious threat to this industry (Prinsloo, 1994; Van Jaarsveld, 1995).

Four different *P. nicotianae* races (race 0, 1, 2 and 3) have been reported to occur on tobacco. These races are defined by their ability to infect various cultivars with different resistance genes (Apple, 1957; Apple, 1962; McIntyre & Taylor, 1978; Prinsloo & Pauer, 1974). Apple (1962; 1967) first described a *P. nicotianae* strain that was non-pathogenic to *Nicotianae plumbaginifolia* Viv. as race 0 and a highly virulent strain as race 1. *P. nicotianae* race 2 is defined by the differential response of 3 cultivars, Kentucky 12 x L8, Burley 21 x L8, and Delcrest 202. Delcrest 202 has a single dominant gene resistant to race 2 (Lamprecht, 1973; Prinsloo & Pauer, 1974; Lamprecht, Prinsloo & Van Wyk, 1974). *P. nicotianae* race 3 is able to overcome resistance in cigar wrapper tobacco and is cold tolerant (Taylor, 1975; McIntyre & Taylor, 1978). It has been suggested that cold tolerance and ketose production are associated with virulence, however, there is no firm genetic data to support this hypothesis (Taylor, 1975; McIntyre & Hankin, 1977).

The continuous large-scale cultivation of tobacco cultivars with resistance to a single race of *P. nicotianae* is not desirable as it increases the selection for new virulent races. For example, cultivation of tobacco cultivars with resistance to race 0 of *P. nicotianae* has, resulted in the appearance of race 1 in North Carolina (Litton, Collins & Legg, 1970; Melton, 1998) and Georgia USA (Csinos & Bertrand, 1994). In South Africa, *P. nicotianae* race 1 appeared in the MKTV trading area after only two years of growing the cultivar Delcrest 202 , which has resistance to race 2 (Lamprecht, 1973; Lamprecht *et al.*, 1974; Prinsloo & Pauer, 1974). The occurrence of *P. nicotianae* race 1 has increased to such an extent in the MKTV area, that Prinsloo (1994) speculated that race 2 is no longer present or occurs at undetectable levels in the soil.

Integrated control programs for black shank include crop rotation, fungicide application and planting resistant cultivars (Melton, 1998). Planting tobacco cultivars with high levels of black shank resistance represents the most economical long-term solution for growers. Five main sources of black shank resistance have been identified in worldwide tobacco breeding programs. They include the cigar tobacco cultivars Florida 301 (Tisdale, 1931; Wernsman, Matzinger & Powell, 1974) and Beinhart 1000-1 (*Nicotianae tabacum* L.) (Silber & Heggestad, 1963; Chaplin, 1966), the flue-cured cultivar Coker 371-Gold (Carlson *et al.*, 1997), *Nicotianae longiflora* Cav. (Valleau,Stokes & Johnson, 1960) and *Nicotianae plumbaginifolia* Viv. (Chaplin, 1962).

South African breeding programs for tobacco cultivars with *P. nicotianae* resistance have been only partially successful (Van Jaarsveld, 1995). Growers have reported an increase in crop loss due to black shank suggesting the appearance of new races or the emergence of more aggressive *P. nicotianae* populations (Van Jaarsveld, 1995). The objectives of this study were, therefore, to assess the variation in aggressiveness in the South African *P. nicotianae* population, to characterize isolates of the pathogen in terms of race, and to assess the resistance to *P. nicotianae* races 0 and 1, in tobacco cultivars commercially deployed in South Africa.

MATERIALS AND METHODS

Isolates

Thirty-eight soil samples were collected at the roots of plants with black shank symptoms. Soil samples were collected at 2-meter intervals in tobacco fields that are known for the occurrence of severe black shank. Soil samples were baited up to three times using citrus leaf discs (Grimm & Alexander, 1973). We also randomly collected 38 infected plants for direct isolation. Infected tissue was surface sterilized in 70% ethanol and rinsed with sterile water. This was followed by the excision and transfer of 6mm square pieces of infected tissue to selective PARPH media (Tsao & Guy, 1977).

After incubation at 26 °C for 2 to 3 days, fungi growing from citrus discs and tobacco tissue were examined. Isolates identified as *P. nicotianae* were sub-cultured onto new plates containing selective media (PARPH) until cultures were clean These *P. nicotianae* isolates were then transferred to potato dextrose agar (Biolab) for aggressiveness studies. All the isolates were identified by morphological characterization of colony characteristics and microscopic examination especially for the presence of papillate sporangia.

The 33 isolates of *P. nicotianae* acquired during this study were obtained as follows: 21 isolates from 4 farms in the Lowveld area, six isolates from 2 farms in the MKTV area, 4 isolates from 1 farm in the PTK area. We also included two isolates, each representing *P. nicotianae* races 2 and 0 as positive controls. The race 0 isolate, P25 (CMW 6917), was obtained from the Kutsaga Tobacco Research Board, Zimbabwe (Masuka & Namichila, 1996), whereas the race 2 isolate, CMW 6914, was supplied by the ARC-Institute for Industrial Crops, Rustenburg, South Africa (Prinsloo & Pauer, 1974). All isolates are maintained in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the Cooperative Research Centre for Tropical Plant Protection, University of Queensland, Australia.

Variation in aggressiveness

Two tobacco cultivars were used to assess variation in aggressiveness of the *P. nicotianae* isolates. These cultivars were TL33 and Hicks. They are moderately tolerant and susceptible, respectively, to black shank (Jones & Mann, 1958; Lamprecht & Prinsloo, 1977).

The *P. nicotianae* isolates used in aggressiveness tests, were incubated on potato dextrose agar (Biolab) at 27 °C in the dark for 5 days. Agar disks (5mm in diameter), taken from the actively growing edges of these cultures were used as inoculum. Four-weeks after planting, TL33 and Hicks tobacco plants were used for these tests. The cambium of the test plants was removed with a corkborer (5mm diam.), 2 cm above ground level. A disc of agar, colonized with *P. nicotianae* was placed into the wound and sealed with parafilm. Eight plants of each cultivar were inoculated with each of the 32 *P. nicotianae* isolates. An equal number of control plants were inoculated with sterile disks of agar. A completely randomized block design was used in the trial. The entire trial was repeated once. In each case the plants were examined for lesion lengths after nine days. Re-isolations were also made from these lesions on a medium containing Pimaricin (Tsao & Ocana, 1969). This was done to verify that the inoculated pathogen was directly responsible for the lesion that developed. Data were statistically analyzed for variance and differences among isolates. Means were tested for significance using Tukey's procedure (Steel & Torrie, 1980).

Race designation

Eight isolates were used for the designation of races. Isolates were selected based on their geographical distribution and relative aggressiveness. These isolates included four isolates from the Lowveld area, two from the MKTV area, one from PTK area and one from Zimbabwe (CMW 6917). A set of seven differential tobacco cultivars that are generally used for race identification (Nielsen, 1995) (Table 1), were inoculated with each of the selected eight *P. nicotianae* isolates as described above. In this test, eight-week old tobacco plants were used and the inoculations, measurements and statistical analyses were performed as described above. Plants were examined for lesion length after seven days.

Evaluation of resistance in commercial cultivars

Three isolates of *P. nicotianae* representing race 0 and three isolates representing race 1 from South Africa were used to evaluate resistance to *P. nicotianae* in 11 commercially used cultivars. Ten flue-cured commercial cultivars were selected from the Lowveld area (LK3/46, LK30/40/60, LK33/60 and Vuma/3/46), the MKTV area (OD1, OD272), Zimbabwe (T20) and North Carolina (Speight G-108, K149 & MDH). A single air-cured cultivar CDL28 from the PTK area was also included. Eight-week-old plants were inoculated with each of the six test isolates. The inoculations, measurements and statistical analyses were performed as described above.

RESULTS

Isolates

P. nicotianae was isolated from all the farms surveyed in this study (Table 2). All these isolates were characterized by the presence of papillate sporangia. Only twenty-five *P. nicotianae* isolates were recovered from the 38 soil samples tested. The reason for this relatively unsuccessful baiting is probably due to applications of fungicides that reduce successful baiting of *P. nicotianae* from soil. Likewise, the use of fungicides on plants may have limited the relative success of isolations from plant tissue. Only eight *P. nicotianae* isolates were recovered from infected plants.

Variation in aggressiveness

Significant differences ($P<0.01$) in lesion length were associated with inoculations with different *P. nicotianae* isolates on Hicks and TL33 (Table 3). Variation in aggressiveness, as reflected by lesion length, was greater on Hicks (6.6 mm to 137.3 mm) than on TL33 (7.5 mm to 64.0 mm). The average lesion length on Hicks (75.1 mm) was significantly higher (LSD cultivars 0.05 = 11.55) than on TL33 (36.9 mm).

A positive correlation was found in the aggressiveness of *P. nicotianae* isolates on the cultivars Hicks and TL33. There were, however, exceptions, since the *P. nicotianae* isolate (CMW 6914; race 2) was characterized as moderately pathogenic on Hicks, but not very pathogenic on TL33. This is probably the presence of *P. nicotianae* race 2-resistance genes in TL33 (Lamprecht *et al.*, 1974; Lamprecht & Prinsloo, 1977). Another exception was *P. nicotianae* isolate CMW 6926, that is highly pathogenic on Hicks, and caused the plants to die through girdling, prior to the development of typical elongated lesions, which were characteristic of the other inoculations. This isolate showed greatest lesion length on TL33. *P. nicotianae* was, furthermore, consistently re-isolated from inoculated plants.

Race designation

Significant differences ($P < 0.05$) were found in lesion lengths associated with various *P. nicotianae* isolates on the differential tobacco cultivars (Table 4). South African *P. nicotianae* isolates were identified as race 0 and race 1 by their differential response of KY14xL8 and Coker371-Gold. Both cultivars are resistant to race 0 and susceptible to race 1 of *P. nicotianae*. The Zimbabwe isolate, CMW 6917 (race 0) had average lesion lengths of 49.0 mm on KY14xL8 and 41.6mm on Coker 371-Gold. The average lesion lengths for isolates CMW 6921, CMW 6916 and CMW 6922 on Coker 371-Gold and KY14xL8 did not differ significantly from those of the Zimbabwe isolate CMW 6917 and were thus identified as race 0 (Table 4). Average lesion lengths for the isolates CMW 6923, CMW 6924, CMW 6925 and CMW 6926 were, however, significantly greater (LSD 0.05 = 26.35) on Coker 371-Gold and KY14xL8 than race 0 isolates and were thus identified as race 1. Race 0 was found in all areas while race 1 was only identified in MKTV and the Lowveld area.

Evaluation of commercial cultivars

Significant differences ($P<0.05$) in lesion length were associated with inoculations of race 0 and 1 *P. nicotianae* isolates on some commercial tobacco cultivars (Table 5). Average lesion length associated with race 1 isolates was significantly greater on OD1 (81.0mm), MDH (70.9mm) and LK33/60 (99.0mm) than with race 0 isolates. Inoculations with race 0

isolates resulted in small lesions on OD1 (50.5mm), MDH (53.7mm) and LK33/60 (57.9mm) reflecting the resistance of these cultivars to race 0. Average lesion length associated with race 0 isolates ranged from 50.5mm (OD1) to 116.5mm (LK30/40/60). For race 1 isolates, average lesion lengths ranged from 37.7mm (LK3/46) to 151.7mm (CDL28). No significant differences ($P<0.05$) were found in lesion length associated with race 0 and 1 isolates on the commercial cultivars Vuma/3/46, T20, OD272, K149 and LK30/40/60.

DISCUSSION

In this study, South African isolates of *P. nicotianae* exhibited significant ($P < 0.05$) differences in aggressiveness to *N. tabacum* cultivars. This is, however, not unusual, since similar results have also been reported elsewhere (Lucas, 1975; Colas *et al.*, 1998). Colas *et al.* (1998) for example showed that within a *P. nicotianae* population from tobacco hosts, large variation in aggressiveness exists between isolates.

Variation in aggressiveness to tobacco plants may lead to a shift in South African *P. nicotianae* populations. This would be similar to the replacement of one genotype of *Phytophthora infestans* by another more aggressive genotype as described in the potato fields in the Columbia Basin, USA (Miller, Johnson & Hamm, 1998). For example, during a tobacco season, the more aggressive isolates of *P. nicotianae* from MKTV and Lowveld area are likely to colonize plant tissue faster. These aggressive isolates might also sporulate faster, thereby increasing their chance of dispersal and dissemination. Aggressive isolates also have the competitive advantage of colonizing a greater number of plants. Plants infected by aggressive isolates may, therefore, have severe black shank symptoms early in the tobacco-growing season. Consequently, less tobacco leaves of high quality would be harvested before plant death.

In this study, we have shown that both races 0 and 1 of *P. nicotianae* occur in South Africa. This is consistent with previous reports of race 0 in the Lowveld and race 1 in the MKTV area (Prinsloo, 1994; Nielsen, 1995). Our results, however, represent the first report of the occurrence of race 0 in the MKTV and PTK area and race 1 in the Lowveld area. The occurrence of races 0 and 1 in these areas is a cause for great concern, since many tobacco cultivars planted here are resistant to only a single race of *P. nicotianae*. Cultivation of

resistant cultivars is likely to result in the emergence of new races of *P. nicotianae* (Apple, 1957; Delon, 1991). Resistance may be overcome by only small changes in existing races due to a mutation in virulence alleles in the *P. nicotianae* population in a similar fashion as reported for the cloned and sequenced virulence allele's of *Cladosporium fulvum* (Joosten *et al.*, 1994). This is consistent with the observation that new races were able to overcome resistance genes in introduced cultivars in Georgia, North Carolina, East Tennessee, and Australia (Gupton, 1972; Csinos & Bertrand, 1994; Robin & Guest, 1994; Melton, 1998).

Significant differences ($P < 0.05$) in resistance or tolerance to both races of *P. nicotianae* were found among the 11 different commercial tobacco cultivars analyzed in this investigation. The extensively planted air-cured cultivar CDL28 was found to be highly susceptible to the pathogen races 0 and 1 whereas the flue-cured cultivars Vuma/3/46 and LK3/46 were highly resistant to both races 0 and 1. Speight G-108, MDH and K149 had moderate resistance to these races as previously reported (Melton, 1993). LK33/60 and OD1 were highly resistant to race 0 but susceptible to race 1.

LK30/40/60 and T20 were rated as susceptible to moderately resistant in this study using the stem inoculation technique. However, previous studies reported that these cultivars had high field resistance to *P. nicotianae* (Nielsen, 1995; Scholtz, 1999). Csinos (1999) proposed that there are at least two types of resistance acting against *P. nicotianae*: root resistance and stem resistance (Hendrix & Apple, 1967). Cultivars, such as T20 and LK30/40/60 have Florida 301 derived resistance that reduces root infection and slows epidemic development (Shew, 1983; Ferrin, & Mitchell, 1986; Shew, 1987). Cultivars with Florida 301 derived resistance, therefore, have low stem resistance to *P. nicotianae* and thus explains the disease response of T20 and LK30/40/60 (Csinos, 1999).

We have shown that both races 0 and 1 occur in tobacco producing regions where these races have not been previously found. The practice of distributing tobacco seedlings from one geographical area to another after severe hail damage may play an important role in the distribution of *P. nicotianae* races in South Africa. Seedlings should be carefully examined for infection by *P. nicotianae* since infected plants have been the major agents of black shank spread in the USA and Australia (Tisdale & Kelley, 1926; Lucas, 1975; O'Brien & Davis, 1981). It is probable or even likely that the occurrence of new races in the Lowveld and MKTV area could be attributed to distribution via seedlings.

Results of this study have provided valuable information on selection of *P. nicotianae* isolates that might be used to augment for future breeding programs in South Africa. Knowledge on the occurrence and distribution of *P. nicotianae* races will assist breeders in the development and deployment of new cultivars. Furthermore, information from this study may be useful in advising farmers on appropriate cultivars to plant in various areas.

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Table 1. Tobacco cultivars used for *P. nicotianae* race identification worldwide

Cultivar	Level of susceptibility	Resistance to *P. nicotianae* Race	Reference
Hicks	High	-	Csinos (1999)
KY 14	Very high	-	Nielsen (1995)
KY 14 x L8	High	0	Nielsen (1995)
Coker 371- Gold	High	0	Carlson *et al.* (1997)
Burley 37	Moderate	0, 1	Collins *et al.* (1971)
NC2326	Low to moderate	0, 1	Wernsman *et al.* (1974), Csinos (1999)
Beinhart 1000-1	Very high	0, 1	Chaplin (1966) Silber & Heggestad (1963)

Table 2. Origin of *Phytophthora nicotianae* isolates collected in this study.

Area	Site	Farm	Number of soil samples	Isolates from soil	Isolates from plants	Total number of isolates
Lowveld	Karino	Barberton Boere	8	5	0	5
		Friedenheim Landgoed	14	11	0	11
	Barberton	Dave Cooper	6	4	0	4
	Schagen	LTGA farm	4	1	0	1
MKTV	Rustenburg	Roelf Otterman	3	1	3	4
		Research Institute for Tobacco and Cotton	0	0	2	2
PTK	Potgietersrus	De Wet Roos	3	2	2	4
Zimbabwe		Kutsaga	0	0	1	1
Total			38	25	8	33

Table 3. Analysis of variance for lesion lengths on two tobacco cultivars (LK33 and Hicks) inoculated with 32 *P. nicotianae* isolates [a]

Source of variation	df	S.S.[b]	F-value	Significance [c]
Treatments [d]	63	8837	12.5	**
Cultivars	1	214943	304.0	**
Isolates	31	8138	11.5	**
Cultivars x Isolates	31	2888	4.1	**
Error	448	707	-	-

[a] Each *P. nicotianae* isolate was inoculated into eight plants of each cultivar

[b] Sum of squares

[c] Significance indicated by ** at $P \leq 0.01$

[d] Treatments consist of all possible combination of 32 *P. nicotianae* isolates and 2 cultivars

Table 4. Lesion lengths (mm) after inoculation with eight *P. nicotianae* isolates on seven differential tobacco cultivars

			Tobacco cultivars[a]						
Isolates	Origin	Race	KY14	Hicks	NC2326	Burley37	KY14xL8	Coker 371 -Gold	Beinhart 1000-1
CMW 6917	Zimbabwe	0	97.37[b] a	48.8 a	43.6 a	46.5 a	49.0 a	41.6 ab	49.6 a
CMW 6922	Lowveld	0	120.3 ab[c]	79.0 b	56.8 ab	46.8 a	64.1 a-d	46.0 a-c	41.5 a
CMW 6921	PTK	0	123.1 a-c	78.3 b	53.5 ab	55.3 a	63.4 a-c	50.1 a-d	36.3 a
CMW 6916	MKTV	0	126.1 b-d	81.0 b	61.5 ab	48.4 a	49.8 ab	40.5 a	39.0 a
CMW 6926	Lowveld	1	128.0 b-e	85.5 b	72.3 b	54.4 a	87.4 c-e	68.8 c-e	45.3 a
CMW 6925	Lowveld	1	147.4 c-f	75.1 b	67.3 ab	55.9 a	119.0 f	82.6 ef	28.1 a
CMW 6924	Lowveld	1	181.5 g	90.8 b	67.6 ab	56.4 a	97.0 ef	96.3 f	33.3 a
CMW 6923	MKTV	1	189.5 g	94.8 b	63.3 ab	58.0 a	96.8 ef	71.6 c-f	45.8 a

[a] KY14 and Hicks are susceptible to *P. nicotianae*, while NC2326 and Burley 37 have low to moderate resistance. KY14xL8 and Coker 371-Gold are resistance to race 0 and susceptible to race 1. Beinhart 1000-1 has the highest resistance to race 0 and 1

[b] Each value represents the mean of 8 replicates

[c] Within each column, mean values followed by the same letter are not significantly different LSD (0.05) =26.35

Table 5. Lesion lengths on 11 commercial tobacco cultivars after inoculation with isolates of *P. nicotianae* race 0 and 1 [a]

Cultivar	*P. nicotianae* isolates								
	Race 0					Race 1			
	CMW 6922	CMW 6921	CMW 6916	AV [b]		CMW 6925	CMW 6924	CMW 6923	AV	
OD1	42.4	54.0	55.3	50.5	ab [c]	64.3	72.3	106.6	81.0	g-l
MDH	58.3	51.9	50.9	53.7	bc	65.1	70.9	76.6	70.9	d-j
LK33/60	51.1	64.9	57.8	57.9	b-e	77.0	105.6	114.5	99.0	m-q
Vuma/3/46	59.9	58.8	62.9	60.5	b-f	58.8	71.3	73.1	67.7	d-g
T20	75.5	55.1	79.8	70.1	d-h	56.8	60.0	56.3	57.7	b-d
LK3/46	78.5	43.9	88.4	70.2	d-i	47.6	33.5	32.0	37.7	a
Speight G-108	62.6	77.0	76.0	71.9	d-k	125.1	90.8	122.3	112.7	p-t
OD272	75.8	82.3	95.5	84.5	h-m	97.3	92.8	79.4	89.8	l-o
K149	111.3	83.5	100.6	98.5	m-p	90.3	84.3	80.9	85.1	h-n
CDL28	91.1	92.1	125.0	102.8	o-r	172.7	158.0	124.4	151.7	u
LK30/40/60	113.6	126.5	109.4	116.5	r-t	110.9	110.0	92.7	104.5	o-s

[a] Each value represents the mean of 8 replicates
[b] Average lesion lengths of 3 *P. nicotianae* isolates
[c] Within each column, mean values followed by the same letter are not significantly different LSD (0.05) = 15.3

Chapter 3

Effect of metalaxyl resistance and cultivar resistance on control of *Phytophthora nicotianae* in tobacco

Van Jaarsveld, E., Wingfield, M. J. & Drenth, A. (2001).
Plant Disease (accepted)

Effect of metalaxyl resistance and cultivar resistance on control of *Phytophthora nicotianae* in tobacco

Phytophthora nicotianae is a devastating root and stem pathogen of tobacco (*Nicotianae tabacum* L.) in South Africa. Growers strive to control the resulting disease, known as black shank, with metalaxyl treatments and resistant cultivars. The aims of this study were to consider whether development of metalaxyl resistance in *P. nicotianae* has contributed to poor disease control and if recently developed cultivars with high levels of resistance require metalaxyl for effective control. One hundred and thirty two isolates of *P. nicotianae* were screened for sensitivity to metalaxyl. *P. nicotianae* isolates from most tobacco farms were metalaxyl sensitive. Growth of most isolates was inhibited completely at 1.0 µg a.i./ml. However, isolates from the MKTV tobacco producing area showed EC50 values ranging from 1.02 µg a.i./ml to 3.57 µg a.i./ml. Twenty-one tobacco cultivars were planted and treated with and without metalaxyl in two different growing seasons to evaluate their resistance to *P. nicotianae* and the effect of using metalaxyl. Hicks was the most susceptible cultivar. Vuma/3/46, LK30/40/60-1 and LK33/60 exhibited the greatest resistance to *P. nicotianae*. Use of metalaxyl in combination with moderately resistant cultivars such as NC60xTL33 and LK10/80/60 effectively reduced black shank in the field. Resistant cultivars were healthy and no significant difference between metalaxyl treated and untreated plants were observed.

Phytophthora nicotianae Breda de Haan (1896) is a devastating root and stem pathogen of tobacco (*Nicotianae tabacum* L.) causing a disease known as black shank. Black shank occurs in most tobacco growing regions in South Africa (Prinsloo, 1994; Van Jaarsveld, 1995). Approximately 25 million kg flue- and 4.8 million kg air-cured tobacco are cultivated annually in the Northern Province (Potgietersrus Co-operative in the PTK trading area), Mpumalanga (Lowveld Golden Leaf Ltd in the Lowveld trading area) and North-West Province (MKTV Co-operative in the MKTV trading area) of South Africa (J. S. Venter, Tobacco RSA, Vermeulen st. 526, Arcadia, Pretoria, South Africa) (Fig. 1).

An integrated black shank disease management program is currently being tested in South Africa. This program is aimed at developing black shank resistant tobacco cultivars, improving the use of fungicides against *P. nicotianae* and implementing crop rotation. This program also incorporates efficient nematode control (Van Jaarsveld, 1995).

Metalaxyl is used worldwide for the control of many Phytophthora diseases (Urech, Schwinn & Staub, 1977; Schwinn, 1983) including black shank (Kannwisher & Mitchell, 1978; Reilly, 1980). Metalaxyl protects tobacco seedlings by inhibiting growth of *P. nicotianae* mycelium and sporangia both in *vivo* and in *vitro* (Staub & Young, 1980; Farih, Tsao & Menge, 1981; Shew, 1984). The fungicide is a xylem-translocated phenylamide with systemic apical movement in plants (Edgington & Peterson, 1977; Cohen & Coffey, 1986). Presently, metalaxyl formulations have 71% of the market share in chemical black shank control in South Africa (SYMPACT Market Information, J. Brits, Crop Product Manager: Subtropical Crops & Cruiser, P. O. Box 92, Isando, 1600, South Africa). Approximately, 12-20% of South African tobacco growers apply metalaxyl (Ridomil 5G® = 50g a.i./kg) routinely before planting at a concentration of 22kg/ha followed by an additional treatment (11kg/ha) 4 weeks later (Van Jaarsveld, 1995). Metalaxyl use in tobacco fields has been associated with the appearance of *P. nicotianae* populations with reduced metalaxyl sensitivity in the USA (Georgia, North Carolina), Korea and Spain (Shew, 1985; Muino, Diaz & Jaenz, 1990; Csinos & Bertrand, 1994; Kim & Kang, 1997). In South Africa, metalaxyl was first introduced into tobacco cultivation during 1978 in the PTK trading area. Two years later, growers in the PTK and MKTV trading areas reported poor black shank control. It has been suggested that this lack of control resulted from accelerated biodegradation of the fungicide (Anonymous, 1999). However, a build up of

resistance to metalaxyl in the *P. nicotianae* population may also give rise to poor disease control. The successful use of metalaxyl in the Lowveld area over a prolonged period of time may be due to the use of more resistant tobacco cultivars in combination with systematic crop rotation (Van Jaarsveld, 1995). Crop rotation in the Lowveld and the PTK region is implemented by cultivating tobacco for one to two years successively. Thereafter, the field is planted with a grass crop (*Rhodes catambora* or *Cenchrus ciliaris*) for two to four years successively. In the MKTV trading area, however, tobacco is cultivated every year in the same fields (Van der Linde, 1992; Prinsloo, 1994).

The aims of this study were to determine (i) whether development of metalaxyl resistance in *P. nicotianae* has contributed to poor disease control and (ii) if recently developed tobacco cultivars with high levels of resistance give better disease control under field conditions in combination with the use of metalaxyl. The outcome of this study would provide useful information to aid implementing an economically viable metalaxyl management program in South Africa.

MATERIALS AND METHODS

Metalaxyl resistance in vitro

One hundred and thirty two *P. nicotianae* isolates were collected from tobacco farms in South Africa, in the PTK, MKTV and the Lowveld area, as well as, from Zimbabwe with 19, 26, 82 and 5 isolates collected, respectively (Table 1). Eighty-nine *P. nicotianae* isolates were obtained from the soil by baiting, with citrus leaves (Grimm & Alexander, 1973). Forty-three *P. nicotianae* isolates were isolated from infected roots and stem tissues of tobacco plants that were surface sterilized in 70% ethanol and then placed on selective PARPH medium (Tsao & Guy, 1977). Cultures were examined for growth of *P. nicotianae* after 3 days and isolates were identified as *P. nicotianae* based on morphology. The cultures have been deposited in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI) (CMW6913-CMW7013 and CMW7906-CMW7938), University of Pretoria, RSA and the Cooperative Research Centre for Tropical Plant Protection, University of Queensland (UQ5253-UQ5373 and UQ5416-UQ5443), Australia.

P. nicotianae isolates were evaluated for their resistance to metalaxyl based on 50 % growth rate inhibition (EC50). The Ridomil® 480 EC formulation (Novartis South Africa) was used to obtain fungicide concentrations of 0.01, 0.1, 1.0 and 10.0 µg a.i./ml (Csinos & Bertrand, 1994). Metalaxyl was added to cool 45 C potato dextrose agar (PDA Biolab South Africa) medium and 12ml of medium was poured per 65mm petri plate. A 5mm-diameter disk was cut from the margin of each fungal isolate growing on PDA for 5 days in the dark at 27 C. Each disk was transferred to the center of an agar plate. Control plates contained only PDA. There were three replicates per isolate at each metalaxyl concentration. Mycelial growth was recorded to the nearest millimeter along the longest radius after 6 days of incubation in the dark at 27 C (Shew, 1985). Average colony diameters were calculated for each isolate at each fungicide concentration. These values were used to calculate EC50 values with the Half-Maximal Response curve for estimating responsiveness in STATISTICA (Statistica, 1993, Statistica for WindowsVersion 4.5B Statsoft Inc. USA.). Each average diameter reading was converted into the percentage of growth inhibited as compared to the control by calculating the difference in the growth between each fungicide concentration, and a curve fitted by STATISTICA. The following equation of the curve was used: $y=b_0-b_0/[1+ (x/b_2)^{b1}]$ where b_0 is the expected response at saturation, b_1 determines the slope of the function, and b_2 is the concentration for a half maximal response. The start value for b_0 was the % inhibition at the highest concentration of metalaxyl for each isolate. Parameter estimates B0, B1 and B2 were calculated by STATISTICA. The concentration at which growth was inhibited by 50% (EC50) was calculated by substituting y=50 into the above equation and solving for x using the parameter estimates. The trial was repeated once.

The effectiveness of metalaxyl and cultivar resistance under field conditions

The field tests were conducted in a naturally infested area with an 8-year history of black shank on the farm Barberton Boere (Table 1). After severe hail in 1997 tobacco season, plants were trimmed back and suckers selected for growing new leaves. However, approximately 80-90% of the plants died of black shank at this site, thus severe black shank occurred over the whole test area. Field tests (1998 and 1999) were conducted at this site for

two consecutive years. Seedling production, fertilization, cultivation, insect control and sucker control followed the Lowveld Tobacco Growers's Association Extension recommendations (Anonymous, 1992; Scholtz, Sonnenberg & Smith, 1998). Seedlings received a Apron® 35 SD (metalaxyl 350 g a.i./kg) treatment applied at 35g/100L water, before transplanting, thus protecting seedlings from *Pythium* and *Phytophthora* species for approximately 4 weeks after planting (Anonymous, 1999). Plots were irrigated as required. The area was plowed and soil thoroughly mixed after each tobacco season Tobacco cultivars used in this study originated from Zimbabwe (T20, Mammoth 10, AW and MS WZ), North Carolina (Speight G-108, Coker 371-Gold, K149, MDH and Hicks), and South Africa (B40/108, NC60xTL33, Vuma, VumaxCoker371-Gold, LK10/80/60, LK3/46, LK30/40/60-1 (selection), LK33/60, LK80/60/60, LK3/46/Vuma, LK30/40/60-2 (selection) and Vuma/3/46).

Twenty-one tobacco cultivars were planted in combination with and without metalaxyl to evaluate fungicide-cultivar combinations for control of tobacco black shank. The experimental design was a randomized complete block design with 4 replicates of 1 row each with 20 plants separated by skip rows. Skip rows were planted with susceptible Hicks plants to ensure continuous inoculum. Plants were spaced 50cm apart within rows and rows were 1.3-m apart. Four replicates received 22kg/ha (50g a.i./kg) metalaxyl (Ridomil 5G®) applied pre-transplanted soil incorporated at a depth of 20cm. The additional four replicates did not receive any chemical treatment and thus served as controls. Nematodes were controlled with Aldicarb (Temik 15G®) applied at 20kg/ha. Plants were examined weekly until all leaves had been harvested. Diseased plants were counted and marked at the first signs of black shank symptoms. The number of diseased plants and the time of symptom development were used to calculate disease indices (Jack & Robertson, 1977). The disease index (DI) was calculated using the following formula:

$DI = [(\sum_{i=1-7} n.w_i / N)] \times 100$

Where n = number of diseased plants for weeks 1 to 7, w = value given for the time of disease onset, w = 1-7 (week 1 = 7, week 2 = 6, week 3 = 5 etc.), N = total number of plants multiplied by the maximum value (w = 7). Plants were considered diseased when the leaves were permanently wilted, stunted, and a black lesion appeared at the base of the

stem. A disease index of 0 indicated no detectable level of disease while that of 100 indicated that all plants had died within the 7-week period.

The entire trial was repeated in two consecutive years. Data were statistically analyzed for differences among cultivars and metalaxyl treatments. Means were tested for significance using Tukey's procedure (Steel & Torrie, 1980).

RESULTS

Metalaxyl resistance in vitro

The range of *P. nicotianae* sensitivity to metalaxyl was determined for individual farms (Table 1). Results showed that the *P. nicotianae* populations from most tobacco farms exhibited low EC50 values and were, therefore, pre-dominantly sensitive to metalaxyl. Growth of most isolates was inhibited completely at 1.0 µg a.i./ml. However, two isolates from the Roelf Otterman farm were slightly resistant to metalaxyl compared to other MKTV isolates (EC50 = 3.57 µg a.i./ml and 1.02 µg a.i./ml). An isolate from the Research Institute for Tobacco and Cotton was also slightly resistant to metalaxyl (EC50 = 0.94 µg a.i. /ml) compared to isolates included in this study.

The mean EC50 values in the Lowveld and PTK area ranged from 0.04 to 0.08 µg a.i. /ml while mean EC50 values in the MKTV area ranged from 0.07 to 0.46 µg a.i./ml (Table 1). *P. nicotianae* isolates from the MKTV appear in general to be slightly more resistant to metalaxyl than isolates from the Lowveld and PTK areas.

The effectiveness of metalaxyl and cultivar resistance under field conditions

High levels of black shank developed in the field as shown by the reaction of the susceptible cultivar Hicks in the skip rows (data not shown) and trials (Table 2). Susceptible plants exhibited black shank symptoms as early as six to seven weeks after planting and included stunting, wilting, and tissue discoloration at the base of the stem. Leaves of plants infected later in the season (ten weeks after planting), turned yellow, wilted, and eventually displayed leaf-bronzing symptoms. Susceptible plants died before topping (week 9 after

planting). Leaves of these plants were immature and could not be harvested. In contrast, most leaves of resistant plants developed normally and were successfully harvested.

Significant differences (LSD_{1998} 0.05 = 6.72, LSD_{1999} 0.05 = 8.22) were found in cultivar resistance as reflected by disease indices (Table 2). Hicks had the greatest disease index since 85.4% and 86.4% of the plants showed black shank symptoms at the end of the 1998 and 1999 seasons when not treated with metalaxyl. The Lowveld cultivars Vuma/3/46, LK30/40/60-1 and LK33/60 had high levels of resistance to *P. nicotianae* with disease indices ranging from 0.3% to 0.9% for the 1998 season and 1.5 % to 11.4% for the 1999 season. Cultivars with moderate resistance to *P. nicotianae* included NC60xTL33 (29% to 29.6%), Vuma (22.8% to 22.9%) and VumaxCoker371-Gold (8.1% to 19.3%). The North Carolina cultivars Speight G-108 (1.3% to 13%) and Coker 371-Gold (4.1% and 9.6%) exhibited high degrees of resistance to *P. nicotianae*. The low disease index values obtained for VumaxCoker 371-Gold and Coker 371-Gold, known for its resistance to race 0 and susceptibility to race 1 (Carlson *et al.*, 1997; Legg & Smeeton, 1999), suggest the presence of race 0 of *P. nicotianae* at this locality. This is in agreement with previous reports of the occurrence of race 0 in the Lowveld region (Nielsen, 1995).

The application of metalaxyl improved the performance of susceptible and moderately resistant cultivars. The use of a single metalaxyl application resulted in significantly (LSD_{1998} 0.05 = 6.72, LSD_{1999} 0.05 = 8.22) lower disease indices (Table 2). Examples include NC60xTL33 (4.4%) and LK10/80/60 (6%) that are moderately resistant and had disease rates of 29.6% and 16.5% respectively. Similarly, the susceptible cultivar Mammoth 10 (35% to 46.7%) had disease index rates of 56.8% to 67.2% without metalaxyl (Table 2). Cultivars such as Vuma/3/46 (0.6% to 1.5%), LK30/40/60-1 (0.9% to 6.9%), MDH (0.3% to 15%) and K149 (0.3%-0. 5%) with high resistance to black shank did not show significant (LSD_{1998} 0.05 = 6.72, LSD_{1999} 0.05 = 8.22) differences in disease indices between metalaxyl treated and untreated plants.

DISCUSSION

Mean EC50 values for *P. nicotianae* isolates examined in this study were within the ranges previously reported for *P. nicotianae* isolates from tobacco (0.01µg a.i./ml to 1.2 µg a.i./ml)

(Shew, 1985; Csinos & Bertrand, 1994) except for one isolate which had an EC50 value of 3.57 µg a.i./ml. Our survey revealed that most *P. nicotianae* populations from the investigated farms in South Africa are currently sensitive to metalaxyl. An exception was found in the MKTV area where two isolates had EC50 values of 1.02 µg a.i./ml and 3.57 µg a.i./ml. Previous authors (Shew, 1985; Csinos & Bertrand, 1994) also reported EC50 values of 1.0µg a.i./ml and 1.2 µg a.i./ml for slightly resistant black shank isolates. Higher EC50 values for metalaxyl resistance have, however, been reported for *P. nicotianae* from other hosts such as citrus (100µg a.i./ml) and periwinkle (742.4µg a.i./ml) (Ferrin & Kabashima, 1991; Timmer, Graham & Zitko, 1998).

Studies conducted by Shew (Shew, 1985) with 877 isolates have shown that continuous application of metalaxyl to field soils infested with *P. nicotianae* resulted in an increase in the EC50 values from 0.4 to 1.2 ug a.i./ml within 3 years. The high EC50 values in comparison to other tobacco areas in South Africa reflects the longer and more intensive use of metalaxyl on these farms. These findings are in agreement with the practice of using metalaxyl every year at the Research Institute for Tobacco & Cotton and the Roelf Otterman farm (MKTV trading area) in comparison with metalaxyl use every two to three years on the farms Barberton Boere, Dave Cooper, Oos Wes Boerdery and the LTGA farm in the Lowveld trading area (Personal communication with growers). Although, this study was limited in sample size (26 isolates) from the MKTV area, this result indicates that a decline in metalaxyl sensitivity may have contributed to poor control of black shank.

The occurrence of slight metalaxyl resistance in South Africa appears to be relatively localized, however, *P. nicotianae* can reproduce very rapidly and metalaxyl resistance in several *Phytophthora* spp. is inherited as a single incomplete dominant gene (Shattock, 1988; Bhat, McBlain & Schmitthener, 1993; Goodwin & McGrath, 1995). Previous studies found that many metalaxyl resistant *P. nicotianae* isolates are inherently fit, virulent and can compete with sensitive isolates (Ferrin & Kabashima, 1991; Timmer *et al.*, 1998). Furthermore, Timmer *et al.* (1998) found that a high percentage of a *P. nicotianae* population remained resistant after 2.5 years without treatment.

Cultivation of susceptible tobacco cultivars in monoculture combined with the continuous use of metalaxyl in South Africa is likely to select for *P. nicotianae* isolates less sensitive to metalaxyl as reported in Georgia and North Carolina (Shew, 1985; Csinos &

Bertrand, 1994). There is no known method for complete eradication of *P. nicotianae* in the field and future monitoring of these populations is required to determine whether metalaxyl resistant isolates become more widespread.

Results of this study show that the use of metalaxyl significantly reduced disease indices thereby improving survival of susceptible and moderately resistant cultivars. This is consistent with findings of Reilly (1980) who also showed that the use of metalaxyl was most effective on tobacco cultivars with little resistance to black shank. For example, a single metalaxyl application in the 1998 season reduced disease incidences of susceptible cultivars such as Mammoth 10 from 56.8% to 46.7% and Hicks from 85.4% to 73.8%.

In this study, highly resistant cultivars such as Vuma/3/46, LK30/40/60 and LK33/60 had low disease indices indicating that plants were healthy and few plants developed black shank symptoms. No significant differences were found between disease indices when resistant plants received a single metalaxyl application. The availability of highly resistant cultivars such as Vuma/3/46, LK30/40/60-1 and LK33/60 to growers provides an opportunity for more effective and economical black shank management. These resistant cultivars have been approved in terms of quality and yield by the tobacco trade in South Africa (Scholtz, 2000). Various factors are required for improved black shank control and increased durability of cultivar resistance. Continued monitoring for spread or increased frequency of *P. nicotianae* isolates with metalaxyl resistance must be a key component of this process. Likewise, it will be important for South African tobacco farmers to use metalaxyl selectively and together with moderately resistant cultivars. The use of highly resistant cultivars, which do not need to be treated with metalaxyl, should be promoted. Efforts to improve the durability of resistance in tobacco cultivars through rotation of cultivars carrying different forms of resistance, as well as improvements in drainage and soil health will be key factors in reducing the impact of this important disease.

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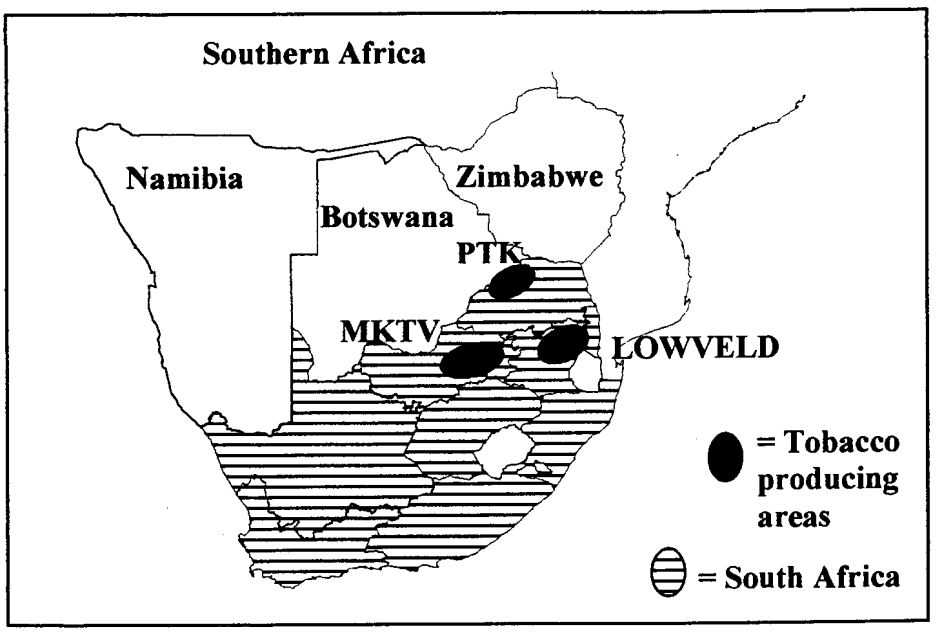

Fig. 1 Map of South Africa showing the different tobacco producing areas (J. S. Venter, Tobacco RSA, Vermeulen st. 526, Arcadia, Pretoria, South Africa).

Table 1. The range and mean metalaxyl EC50 for 132 isolates of *Phytophthora nicotianae.*

Area	Site	Farm	Number of Isolates	Metalaxyl EC50 [1] Min	Max	Mean
Lowveld	Karino	Barberton Boere	12	0.01	0.15	0.06
		Friedenheim	45	0.00	0.32	0.04
	Barberton	Dave Cooper	16	0.00	0.46	0.08
	Schagen	Oos Wes Boerdery	3	0.03	0.12	0.06
		LTGA farm	6	0.02	0.14	0.06
MKTV	Rustenburg	Roelf Otterman	17	0.07	3.57	0.46
		Research Institute for Tobacco & Cotton	7	0.03	0.94	0.26
	Naboomspruit	A. Botha	2	0.01	0.14	0.07
PTK	Potgietersrus	De Wet Roos	14	0.00	0.63	0.06
	Levubu	A. Botha	5	0.03	0.09	0.06
Zimbabwe		Kutsaga	5	0.00	0.57	0.15
Total			132	0.0001	3.565	0.123

[1] EC50 is in µg a.i./ml

Table 2. Comparison of disease indices linked to susceptibility to *P. nicotianae* for 21 tobacco cultivars grown - with and without metalaxyl

	Disease Index [1]							
	1998				1999			
Cultivars	Without Metalaxyl		With Metalaxyl		Without Metalaxyl		With Metalaxyl	
Susceptible								
Hicks	85.4	cc[2]	73.8	z-bb	86.4	LL[3]	69.3	GG-JJ
AW	67.9	x-z	66.4	xy	80.3	KKLL	66.5	GG
MS WZ	70.0	x-aa	64.0	x	74.4	GG-KK	66.7	GGHH
Mammoth 10	56.8	w	46.7	v	67.2	GG-II	35.0	EEFF
Moderately Resistant								
NC60xTL33	29.6	u	4.4	a-k	29.0	AA-EE	4.5	A-G
Vuma	22.8	st	14.7	p-r	22.9	W-CC	14.4	M-V
LK10/80/60	16.5	q-s	6.0	a-m	16.6	N-Y	6.1	A-L
T20	6.6	a-n	0.6	a-c	22.7	V-BB	15.1	M-X
VumaxCoker371-Gold	8.1	f-p	6.6	a-n	19.3	S-Z	9.3	C-N
LK3/46	12.3	m-q	5.2	a-l	26.2	Z-DD	21.9	V-AA
Highly Resistant								
MDH	0.3	ab	3.8	a-i	15.0	M-W	10.2	D-R
Speight G-108	1.3	a-e	1.6	a-f	13.0	H-U	5.4	A-I
Coker371-Gold	4.1	a-j	6.9	b-o	9.6	C-O	9.7	C-P
LK30/40/60-1	0.9	a-d	0.0	a	6.9	A-M	4.9	A-H
LK33/60	0.3	ab	0.9	a-d	11.4	F-T	5.6	A-J
LK80/60/60	1.3	a-e	0.0	a	9.8	D-P	10.0	D-Q
LK3/46/Vuma	3.4	a-h	1.9	a-g	5.8	A-K	3.0	A-E
LK30/40/60-2	1.9	a-g	0.0	a	11.1	E-S	4.3	A-F
B40/108	0.3	ab	0.0	a	2.4	A-D	0.5	AB
Vuma/3/46	0.6	ac	0.0	a	1.5	A-C	0.0	A
K149	0.3	ab	0.0	a	0.5	AB	0.0	A

[1]The disease index was calculated using the formula: $DI = [(\sum_{i=1-7} n.wi / N)] \times 100$ Where n = number of diseased plants for weeks 1 to 7, w = value given for the time of disease onset w = 1-7 (week 1 = 7, week 2 = 6, week 3 = 5 etc.), N = total number of plants multiplied with the maximum value (w = 7). A disease index of 0 indicated no detectable level of disease while that of 100 indicated that all plants had died within the 7-week period. Each value represents the mean of 4 replicates.

[2] Within columns for the 1998 season, values followed by the same letter are not significantly different according to Tukey's LSD (0.05) = 6.72

[3] Within columns for the 1999 season, values followed by the same letter are not significantly different according to Tukey's LSD (0.05) = 8.22

Chapter 4

A rapid seedling based screening technique to assay tobacco for resistance to *Phytophthora nicotianae*

A rapid seedling based screening technique to assay tobacco for resistance to *Phytophthora nicotianae*

Black shank caused by *Phytophthora nicotianae* is a serious root and stem disease of cultivated tobacco worldwide. In this study, a rapid seedling based screening technique was developed to evaluate tobacco cultivars for resistance to *P. nicotianae*. This technique was compared with a stem inoculation technique commonly used on adult plants. The overall aim was to develop an improved and rapid technique that could also be used to characterize races of *P. nicotianae*. Six cultivars (Coker 371-Gold, KY14, KY14xL8, Beinhart 1000-1, Vuma/3/46 and LK33/60) were inoculated with three isolates each of race 0 and 1. Seedlings at the four-leaf stage were transferred to floating trays and inoculated at the roots with a zoospore suspension. Three days after inoculation, seedlings were evaluated using a disease rating scale. For stem inoculations, adult plants were inoculated in the stems with mycelium plugs. A strong positive correlation was found between results of the seedling assay and adult plant trials for all isolates and cultivars tested. Furthermore, *P. nicotianae* isolates could be characterized as race 0 or 1 using both stem inoculation and the rapid seedling assay. This assay will facilitate rapid and large scale screening for black shank resistance and therefore has the potential to reduce yield losses due to this diseases in the field in South Africa.

Phytophthora nicotianae is an important root and stem pathogen in South Africa where it causes a disease known as black shank on tobacco (Van Jaarsveld, 1995). Symptoms of black shank include wilting and stunting of plants, as well as characteristic black lesions at the bases of the stems (Lucas, 1975; Shew, 1991). For effective black shank control, growers use a combination of crop rotation, cultivar resistance and fungicide applications (Melton, 1998). Fungicides such as metalaxyl, are however expensive and growers tend to use lower than optimum dosages (Csinos & Bertrand, 1994), which may contribute to the development of metalaxyl resistant *P. nicotianae* populations in South Africa, Korea and the USA (Kim & Kang, 1997; Shew, 1985; Van Jaarsveld, Wingfield & Drenth, Chapter 3).

Breeding and the deployment of resistant cultivars are the most economical approaches to controlling *P. nicotianae.* Modern flue-cured cultivars are derived from 5 main sources of resistance to race 0 and 1 of the pathogen. They include cultivars Florida 301 and Beinhart 1000-1 in *Nicotianae tabacum* with partial resistance to race 1 and 0 (Tisdale, 1922; Silber & Heggestad, 1963); Coker 371-Gold with resistance to race 0 (Carlson *et al.*, 1997), as well as, *Nicotianae longiflora* (Burley cultivar L8) and *Nicotianae plumbaginifolia* with single dominant gene resistance to race 0 (Chaplin, 1962; Valleau, Stokes & Johnson, 1960).

The selection of cultivars and breeding lines for resistance to *P. nicotianae* has traditionally been conducted in fields where the pathogen is known to occur. Field trials are, however; labor intensive, expensive and time consuming. Furthermore, the inoculum is commonly not evenly distributed and results can thus be equivocal. More than one race of *P. nicotianae* may also occur simultaneously in a tobacco field as reported in South Africa and the USA, which results in further confusion (Melton, 1998; Van Jaarsveld, Wingfield & Drenth, Chapter 2).

A variety of techniques have been developed to screen tobacco germplasm for resistance to *P. nicotianae.* These include root, stem and leaf inoculations (Apple, 1957; Hendrix & Apple, 1967; Litton, Legg & Collins, 1970; Tedford, Miller & Nielsen, 1990; Rufty, Wernsman & Gooding, 1987). Root and stem inoculation trials may be limited by the availability of space in greenhouses. Leaf inoculations are non-destructive but they are limited in the detection of resistance expression (Tedford *et al.*, 1990). Maia *et al.* (1995) developed two seedling-screening techniques. The first of these utilized young plants

sprayed with zoospores at the cotyledon stage and the second technique relied on inoculating seedling roots with mycelium. These two techniques have shown that both specific and non-specific resistance can be detected on very young plants (Maia *et al.*, 1995). The aim of the present study was to develop an improved rapid screening technique based on the method of Maia *et al.* (1995). Results using this technique were then compared with those from stem inoculations on adult plants in the greenhouse. The potential to use this screening technique for race characterization was also considered.

MATERIALS & METHODS

Fungal isolates and tobacco cultivars

Four differential and two commercial tobacco cultivars were used in this study. The differential cultivars, KY14, KY14xL8, Coker 371-Gold and Beinhart 1000-1 were selected based on their specific resistance genes and respective susceptibility to race 0 and 1 of *P. nicotianae* (Nielsen, 1995). KY14 is highly susceptible while Beinhart 1000-1 is the most resistant cultivar to both races (Chaplin, 1966; Silber & Heggestad, 1963). KY14xL8 and Coker 371-Gold are resistant to race 0 but susceptible to race 1 (Carlson *et al.*, 1997; Csinos, 1999). The two flue-cured commercial cultivars used, were LK33/60 and Vuma/3/46.

P. nicotianae isolates previously characterized as race 0 and 1 (Van Jaarsveld *et al.* Chapter 2) were selected for this study. These included: three isolates from the Lowveld (Mpumalanga), one from PTK (Northern Province) and two from the MKTV (North-West Province) tobacco trading areas in South Africa (Table 1). The cultures used for inoculation have been deposited in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, RSA and the Cooperative Research Centre for Tropical Plant Protection, University of Queensland, Australia.

Inoculum production

P. nicotianae cultures were grown for five days on potato-dextrose agar at 27 °C in the dark. Four 6mm discs taken from the actively growing margins of fresh cultures were placed in each of five Petri dishes (65mm diam) containing 12ml sterile Pea broth solution (Ribeiro, 1978) amended with 0.01g ß-sitosterol (Sigma AnalaR) and incubated at 28 °C for 2 days with continuous light. After incubation, mycelial mats were washed once with sterile Petri's salt solution (Ribeiro, 1978) amended with 0.002g E.D.T.A. Disodium salt (Holpro Lovasz, South Africa) and 0.002g $MnSO_4$ (ACE, South Africa). Mycelial mats were incubated at 28°C under continuous light in Petri's salt solution (12ml Petri's salt solution in each Petri dish). After 48h, the mycelial mats were harvested and cultures were examined using a light microscope to confirm the presence of sporangia and the production of zoospores.

Seedling inoculations

Tobacco seeds were germinated on Hygrotech seedling medium consisting of sphagnum peat moss and medium grade vermiculite (pH 7). After approximately 3 weeks, seedlings at the 4-leaf stage were selected. Seedlings of each cultivar were removed from the seedbeds and gently rinsed with dH_2O to remove potting mixture from the roots. Individual seedlings were transferred into polystyrene trays with small cavities (3mm diam) (Figs. 1-2). Each seedling was individually placed in a cavity in the polystyrene trays, with the leaves of the seedling arranged above the tray surface and the roots hanging through the drainage hole below the tray. Trays containing seedlings were then transferred to 3L plastic containers. Each plastic container contained a suspension of 100ml sterile Petri's salt solution, 100ml sterile dH_2O and 60ml *P. nicotianae* inoculum (7.2×10^4 zoospores/ml). The polystyrene trays with seedlings floated on the suspension in the plastic containers. Thereafter, seedlings were sprayed lightly with dH_2O and the plastic containers containing the polystyrene trays with seedlings were placed in clear plastic bags in order to maintain a high level of humidity. The plastic containers with seedlings and inoculum were maintained at room temperature (28 °C to 30 °C) for 3 days.

Ten seedlings of each tobacco cultivar (Coker 371-Gold, KY14, KY14xL8, Beinhart 1000-1, Vuma/3/46 and LK33/60) were placed in a polystyrene tray. Two polystyrene trays containing all cultivars were inoculated with each of the six *P. nicotianae* isolates. Seedlings of tobacco cultivars were placed in a completely randomized block design in the trays. In each case the seedlings were examined for symptoms using a scale (Table 2) that reflects different levels of infection. This disease rating scale is based on Maia *et al.* (1995) disease index for seedlings. For controls, a separate plastic container of seedlings were floated on distilled water free of *P. nicotianae* inoculum. Re-isolations from infected plants were performed on PARPH medium (Tsao & Guy, 1977) to verify that symptoms were due to the effect of the inoculated pathogen. The entire experiment was repeated once and results were compared with those from stem inoculations on adult plants in the greenhouse.

Adult plant inoculations

The six *P. nicotianae* isolates selected for this study were grown on potato dexstrose agar (Biolab) at 27°C in the dark for five days. Agar disks (5mm in diam), taken from the actively growing edges of these cultures were used as inoculum for pathogenicity tests. Eight weeks after planting, tobacco plants were inoculated by removing the cambium from the stems of the test plants using a corkborer (5mm diam), 2cm above ground level. A disc of agar, colonized with *P. nicotianae* was placed into the wound, mycelium side downwards and sealed with parafilm.

Eight plants of each tobacco cultivar (Coker 371-Gold, KY14, KY14xL8, Beinhart 1000-1, Vuma/3/46 and LK33/60) were inoculated with each of the six *P. nicotianae* isolates. An equal number of control plants were inoculated with sterile disks of agar. A completely randomized block design was used in the trial and the entire trial was repeated once. In each case the plants were examined for lesion length seven days after inoculation. Re-isolations were made from the lesions on PARPH medium (Tsao & Guy, 1977) to verify that the inoculated pathogen had caused the lesion.

Validation of the seedling assay

Data obtained from the seedling and adult plant inoculations were statistically analyzed for variances and differences among isolates and tobacco cultivars. Means were tested for significance according to Tukey's procedure (Steel & Torrie, 1980). A linear regression was computed to compare results of the stem inoculation and the seedling assay.

RESULTS

Seedling inoculations

Symptoms developed on susceptible seedlings 48hr after inoculation with *P. nicotianae*. For example, KY14 seedlings inoculated with isolate CMW 6924 exhibited yellow leaves, brown stems and root rot symptoms (Fig. 1). Nine of the latter ten KY14 seedlings were dead after 3 days and had disease rating scale values of 5 (Table 3). In contrast, seedlings with resistance to *P. nicotianae*, such as the ten Beinhart 1000-1 seedlings inoculated with isolate CMW 6922 were all green and healthy after 3 days and had disease rating scale values of 1 (Table 3).

Significant differences (LSD 0.05 = 0.85) were found for disease ratings associated with the various *P. nicotianae* isolates on the differential and commercial cultivars tested (Table 3). The seedling assay allowed us to effectively distinguish between susceptible seedlings of KY14 where disease ratings ranged from 3.10 to 4.95 and resistant seedlings of Beinhart 1000-1 where values ranged from 1.00 to 2.00. All seedlings of Beinhart 1000-1 inoculated with either race of the pathogen survived. The commercial cultivars LK33/60 and Vuma/3/46 were more susceptible to race 1 where disease ratings ranged from 3.10 to 5.00 than race 0 where disease ratings ranged from 1.10 to 3.45.

P. nicotianae isolates could be distinguished as race 0 and 1 based on the response of the cultivars KY14xL8 and Coker 371-Gold to infection. Disease ratings of KY14xL8 and Coker 371-Gold seedlings inoculated with race 1 isolates ranged from 3.85 to 5.00 and did not differ significantly (LSD 0.05 = 0.85) from the susceptible cultivar KY14, where values ranged from 3.10 to 4.95. However, when seedlings of KY14xL8 and Coker 371-

Gold were inoculated with race 0 isolates, disease ratings ranged from 1.65 to 2.60 and differed significantly (LSD 0.05 = 0.85) from KY14 where values ranged from 3.00 to 4.45. The disease ratings of the cultivars KY14xL8 and Coker 371-Gold infected with race 0 and 1 isolates thus reflected the well established resistance of these cultivars to race 0 of *P. nicotianae*.

Adult plant inoculations

In stem inoculation trials, symptoms on susceptible adult plants included wilting and rapid collapse of the stem tissue. This resulted in black lesions at the bases of stems, which is characteristic of black shank (Fig. 3). Adult plants with resistance to *P. nicotianae* remained green and healthy with only small lesions at the points of inoculation (Fig. 4).

Significant differences (LSD 0.05 = 31.36) were found in lesion lengths associated with the various *P. nicotianae* isolates on differential and commercial cultivars (Table 4). Lesion lengths on the resistant cultivar Beinhart 1000-1 were significantly (LSD 0.05 = 31.36) smaller (28.1mm to 45.8 mm) than those on susceptible cultivar KY14. All adult Beinhart 1000-1 plants inoculated with race 0 and 1 survived. Lesion lengths on the commercial cultivars LK33/60 and Vuma/3/46 showed that they have higher levels of resistance to race 0 with lesions ranging from 51.1mm to 64.9mm compared to those associated with race 1 ranging from 58.8mm to 114.5mm.

P. nicotianae isolates could be distinguished as race 0 and 1 based on the lesion lengths on cultivars KY14xL8 and Coker 371-Gold. Lesions on KY14xL8 and Coker 371-Gold inoculated with race 1 isolates ranged from 71.6mm to 119.0mm and did not differ significantly (LSD 0.05 = 31.36) from those on the susceptible cultivar KY14, where lesions ranged from 147.4mm to 189.5mm long. However, when adult plants of KY14xL8 and Coker 371-Gold were inoculated with race 0 isolates, lesion lengths ranged from 40.5mm to 64.1mm and differed significantly (LSD 0.05 = 31.36) from KY 14 where these values ranged from 120.3mm to 126.1mm. Lesion lengths on KY14xL8 and Coker 371-Gold plants inoculated with race 1 were significantly larger than those caused by race 0 thus reflecting the established resistance of these cultivars to race 0.

Validation of the seedling assay

A positive correlation ($R^2 = 0.68$) was found between the average lesion lengths on adult plants and disease ratings (symptom scales) on seedlings. This was true for all 6 *P. nicotianae* isolates and all cultivars tested (Fig. 5). Thus plants with resistance to *P. nicotianae* could be reliably identified using the seedling assay, as well as, the stem inoculation technique.

The difference between *P. nicotianae* isolates belonging to race 0 or 1 could be identified as using both the stem inoculation technique and the rapid seedling assay. Disease ratings on seedlings and average lesion lengths (Tables 3 & 4) for isolates CMW 6921, CMW 6916 and CMW 6922 were significantly lower ($P < 0.05$) in the cultivars KY14xL8 and Coker 371-Gold, which are known to have resistance to race 0 than the susceptible cultivar KY14. Isolates CMW 6921, CMW 6916 and CMW 6922 were thus characterized as race 0 of *P. nicotianae*. Isolates CMW 6923, CMW 6924 and CMW 6925 were likewise characterized as race 1 since these isolates had high disease ratings and longer lesions on Coker 371-Gold and KY14xL8.

DISCUSSION

In this study, a rapid seedling-based technique was developed to identify and characterize plants with resistance to black shank. This can now be effectively used in screening F1 progeny from breeding trials. Resistance identified using this seedling assay was positively correlated with that identified with the more commonly used adult plant inoculation technique (Hendrix & Apple, 1967; Wills, 1971; Wills & Moore, 1971).

Using the rapid screening technique developed in this study, the commercial cultivar Vuma/3/46 was rated as more susceptible to race 1 than with the stem inoculation technique. Here, it must be recognized that the two techniques are fundamentally different with one linked to natural root infection and the other to stem wound infection. When this difference is considered, the result is not unusual and previous authors (Hendrix & Apple, 1967; Csinos, 1999) have also reported differences between stem and root resistance to *P.*

nicotianae. Thus the cultivar Vuma/3/46 exhibits high levels of stem resistance in adult plants while it displays a level of susceptibility in seedling roots.

The rapid screening assay, in combination with the differential cultivars, was effective in characterizing *P. nicotianae* isolates as belonging to race 0 and 1. This technique could potentially be used to identify *P. nicotianae* races on commercial tobacco farms. Knowledge of the races of *P. nicotianae* present on commercial farms is valuable to extension officers who must recommend to growers, cultivars with appropriate resistance.

The seedling assay presented in this study has been used successfully in the characterization of large numbers of disease resistant F1 progeny in breeding trials at the Lowveld Tobacco Growers Association (Mpumalanga) in South Africa. The technique provides rapid results and is inexpensive. Using a zoospore suspension as inoculum has reduced the incubation period for seedlings to 3 days. This is in contrast to the technique of Maia *et al.* (1995) where seedlings were incubated for 7 days. The use of polystyrene trays and plastic containers is also less tedious than transferring seedlings and *P. nicotianae* mycelium to individual Eppendorf tubes as described by Maia *et al.* (1995).

The limitation of the seedling assay described in this study is that is difficult to select seedlings with low or moderate resistance to infection. Maia *et al.* (1995) emphasized the importance of choosing isolates of *P. nicotianae* with intermediate levels of pathogenicity that will kill all susceptible cultivars, but not those with low levels of resistance. Adult plant screening of wild germplasm and parental material should not be discarded from breeding programs since not all resistance may express itself at the seedling stage.

The ability to screen large numbers of tobacco plants rapidly at the seedling stage is an essential component of effective black shank resistant breeding programs. It allows for the testing of large germplasm resources in a systematic manner and under standard conditions. Ultimately, this will promote the availability of new black shank resistant cultivars for commercial deployment. Financially, growers will benefit from improved yields and higher quality plants as well as lower production costs.

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Figs. 1-4 Seedling and adult tobacco plants inoculated with *Phytophthora nicotianae*. **Fig. 1** The rapid seedling based screening technique exhibiting susceptible seedlings with yellow leaves, brown stems and root rot symptoms (arrow) after three days. **Fig. 2** The rapid seedling based screening technique exhibiting healthy green seedlings that serve as the control. **Fig. 3** A tobacco plant exhibiting the characteristic black lesion (arrow) after stem inoculation with *P. nicotianae*. **Fig. 4** A tobacco plant with resistance to *P. nicotianae* remained green and healthy with only a small lesion (arrow) at the point of inoculation.

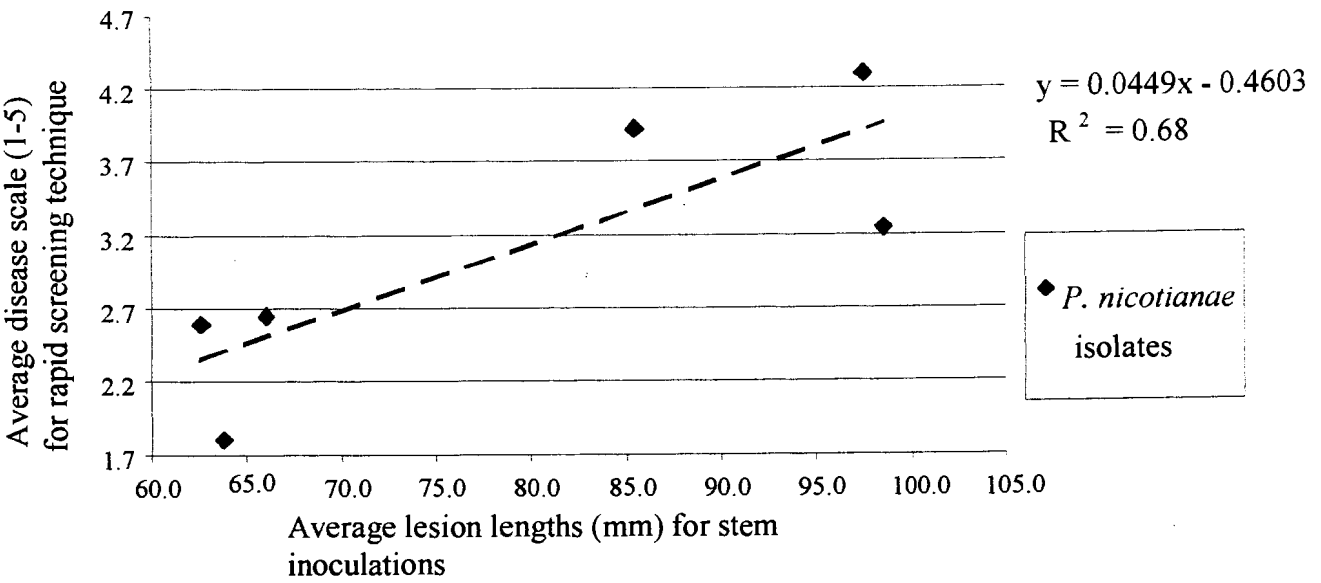

Fig. 5 Comparison of rapid screening technique and stem inoculations for 6 *Phytophthora nicotianae* isolates.

Table 1. *P. nicotianae* isolates used in this study

Ascession number	Mating type	*P. nicotianae* race	Tobacco trading areas in South Africa
CMW 6921	A1	0	PTK
CMW 6916	A2	0	MKTV
CMW 6922	A1	0	Lowveld
CMW 6923	A2	1	MKTV
CMW 6924	A1	1	Lowveld
CMW 6925	A1	1	Lowveld

Table 2. Disease rating scale for symptoms on seedlings after infection by *P. nicotianae*

Disease scale	Symptoms [1]
1	Healthy green seedlings
2	Bottom leaves turn light yellow
3	Bottom and middle leaves are yellow
4	Seedlings are damping off, all leaves are yellow and stems are brown
5	Seedlings dead

[1]Symptoms evaluated three days after inoculation of *P. nicotianae*

Table 3. Disease rating scale values of 6 tobacco cultivars after inoculation with 6 *P. nicotianae* isolates [1]

Cultivars	*P. nicotianae*					
	Race 0			Race 1		
	CMW 6921	CMW 6916	CMW 6922	CMW 6923	CMW 6924	CMW 6925
Differential cultivars						
KY14	4.45 e [2]	3.00 cd	3.10 d	3.10 b	4.95 b	3.65 b
KY14xL8	2.00 ab	1.80 a	2.05 c	4.00 c	4.90 b	5.00 d
Coker 371-Gold	2.60 a-d	2.40 a-c	1.65 a-c	3.85 bc	4.65 b	4.10 bc
Beinhart 1000-1	1.80 a	2.00 ab	1.00 a	1.75 a	1.80 a	2.00 a
Commercial cultivars						
LK33/60	2.95 cd	2.90 cd	1.95 bc	3.70 bc	5.00 b	4.60 cd
Vuma/3/46	2.10 ab	3.45 d	1.10 ab	3.10 b	4.50 b	4.20 b-d

[1] Each value represents the mean of 10 replicates.

[2] Within each column, values followed by the same letter are not significantly different Tukey LSD (0.05) =0.85

Table 4. Lesion lengths on 6 tobacco cultivars after inoculation with 6 *P. nicotianae* isolates [1]

Cultivars	*P. nicotianae*					
	Race 0			Race 1		
	CMW 6921	CMW 6916	CMW 6922	CMW 6923	CMW 6924	CMW 6925
Differential cultivars						
KY 14	123.1 b [2]	126.1 b	120.3 b	189.5 e	181.5 e	147.4 e
KY 14xL8	63.4 a	49.8 a	64.1 a	96.8 b-d	97.0 b-d	119.0 de
Coker 371-Gold	50.1 a	40.5 a	46.0 a	71.6 a-b	96.3 bc	82.6 b-d
Beinhart 1000-1	36.3 a	39.0 a	41.5 a	45.8 a	33.3 a	28.1 a
Commercial cultivars						
LK33/60	64.9 a	57.8 a	51.1 a	114.5 d	105.6 b-d	77.0 bc
Vuma/3/46	58.8 a	62.9 a	59.9 a	73.1 a-c	71.3 ab	58.8 ab

[1] Each value represents the mean of 8 replicates, lesion lengths are measured in the cambium (mm).

[2] Within each column, values followed by the same letter are not significantly different Tukey LSD (0.05) =31.36

Chapter 5

Genotypic diversity of *Phytophthora nicotianae*, the causative organism of black shank on tobacco in South Africa

Genotypic diversity of *Phytophthora nicotianae*, the causative organism of black shank on tobacco in South Africa.

Phytophthora nicotianae has been the cause of considerable quality and yield loss due to root and stem disease in the citrus and tobacco industries of South Africa. One hundred and five *P. nicotianae* isolates were collected from the Northern Highveld and Lowveld region, from both citrus and tobacco hosts in South Africa. We identified *P. nicotianae* isolates using restriction fragment length polymorphism's of an amplified fragment of the ribosomal DNAs (rDNA), as well as, DNA sequence information from the rRNA internal transcribed spacers regions (ITS 1 and ITS 2). Based on ITS sequence analysis citrus and tobacco isolates clustered together with *P. nicotianae*. Thirty-nine polymorphic RAPD fragments from five decanucleotide Operon primers revealed 79 different RAPD phenotypes among a total of 105 *P. nicotianae* isolates analysed. Thirty-five of the 105 *P. nicotianae* isolates were clonal. These clonal *P. nicotianae* isolates were from both citrus and tobacco hosts and occurred in Northern Highveld and Lowveld region. Lowveld *P. nicotianae* isolates were separated into 54 and Northern Highveld isolates into 25 different RAPD phenotypes. *P. nicotianae* isolates from citrus hosts separated into 12 different RAPD phenotypes while *P. nicotianae* isolates from tobacco hosts separated into 67 different RAPD phenotypes. The high number of RAPD phenotypes (79) in relation to the sample size (105), the presence of both the A1 and A2 mating type and high levels of phenotypic diversity in the *P. nicotianae* population indicate a sexually propagating population in South Africa. Reciprocal inoculations with isolates from tobacco and citrus furthermore showed that isolates from tobacco were as pathogenic as citrus isolates on the citrus cultivar Rough Lemon while isolates from tobacco were more pathogenic on tobacco cultivar Coker 371-Gold than citrus isolates.

The soilborne pathogen *Phytophthora nicotianae* Breda de Haan is the causative agent of root and stem disease to over 72 different plant genera (Hickman, 1958). In South Africa, this pathogen is associated with considerable quality and yield loss in the citrus (Thompson, Phillips & Nel, 1995) and tobacco industries (Van Jaarsveld, 1995). Both these crops are economically important and the gross incomes from agricultural production of citrus and tobacco during 1999 amounts to 1.8 billion Rand for citrus (H. F. le Roux, Outspan Citrus Center, Nelspruit, pers. comm.) and 423 million Rand for tobacco (J. S. Venter, Tobacco RSA, pers. comm.). *P. nicotianae* is an important root pathogen of citrus since 65% of the orchards in South Africa are infested with this pathogen (Le Roux *et al.*, 1991) and root disorders causes a R90 million loss annually (Phillips, 1994). In order to reduce crop losses due to *P. nicotianae* infestation, disease control measures including fungicide application, alternative cultural practices and resistance breeding have been applied (Le Roux *et al.*, 1991; Van Jaarsveld, 1995).

P. nicotianae is a formidable pathogen in South African tobacco cultivation and it is commonly found in the flue- and air-cured tobacco-growing regions in the Northern Highveld (Potgietersrus Co-operative and MKTV Co-operative in the Northern and North-West Province) and the Lowveld (Lowveld Golden Leaf Pty. in Mpumalanga) (Fig.1) (Van Jaarsveld, 1995). Furthermore, there is evidence suggesting that new races of the pathogen have evolved in South Africa (Van Jaarsveld, Wingfield & Drenth, Chapter 2) which overcome the resistance genes used in widely grown tobacco cultivars. Breakdown of resistance has also been suggested for *P. nicotianae* in North Carolina and Georgia in the USA (Lucas, 1975; Csinos & Bertrand, 1994).

P. nicotianae is an heterothallic Oomycete and individuals display either one of two (A1 or A2) mating types. These individuals of opposite mating type can interact and sexually reproduce through the formation of oospores, which may survive for many years in soil. Currently, it is unknown whether both mating types are present in the South African *P. nicotianae* populations. Förster & Coffey (1990) found preliminary evidence of *in vitro* sexual recombination in *P. nicotianae* oospores with a high degree of variation in growth rate and colony morphology. However, the role of sexual reproduction in *P. nicotianae* and its effect on levels of genotypic diversity in the natural environment as well as under agronomic conditions, is unknown. Sexual recombination gives rise to new allele

combinations and the generation of novel phenotypes, which potentially may overcome combinations of cultivar resistance genes more quickly compared to purely asexual reproducing pathogen populations (McDonald & McDermott, 1993). Resistance to fungicides may also build up to higher levels in these populations more quickly (Drenth, Tas & Govers, 1994).

Diversification in South African agriculture has resulted in many growers cultivating tobacco adjacent to citrus orchards or alternatively, converting old citrus orchards into tobacco fields. It is generally thought that *P. nicotianae* isolates are host specific (Apple, 1957; Apple, 1962; Lucas, 1975; Shew, 1991) and that isolates from citrus orchards cannot infect the newly planted tobacco crops. However, as far as we are aware, this aspect of the biology of *P. nicotianae* has not been investigated and no solid evidence excists to substantiate this claim.

Despite the agricultural and economic importance of *P. nicotianae* in many crop species, few studies have been conducted on the population genetic structure of this pathogen. One study of 87 *P. nicotianae* isolates collected from 25 different countries revealed eight mtDNA haplotypes (Lacourt *et al.*, 1994). The predominant mtDNA haplotype had the widest geographical distribution (Lacourt *et al.*, 1994) and it was proposed that the present *P. nicotianae* population is derived from one dominant mtDNA lineage (Lacourt *et al.*, 1994). However, mtDNA does not reveal the amount and distribution of genetic diversity in the nuclear genome and it is not involved in sexual recombination. A few population studies using DNA-fingerprinting techniques based on multicopy RFLP probes and random amplified polymorphic DNA (RAPD's) have been used to determine levels of genotypic diversity in other *Phytophthora* species (Goodwin *et al.* 1992; Fry *et al.*, 1993; Linde, Drenth & Wingfield, 1999). Detailed population genetic studies of *P. nicotianae* populations could reveal valuable insight into the mode of reproduction, as well as, the distribution of clonal genotypes and genetic differentiation based on either host or geography.

The specific aims of this study were to; (i) determine the similarity of *P. nicotianae* strains from tobacco and citrus from South Africa to each other and compare them to published reference strains (Cooke *et al.* 2000) from elsewhere using ribosomal DNA sequences, (ii) determine whether isolates of *P. nicotianae* obtained from citrus and tobacco

belong to the same pathogen population and are able to cross infect their respective hosts, (iii) test for the presence and distribution of A1 and A2 mating types of *P. nicotianae* in tobacco fields, (iv) assess the level of genotypic diversity in the *P. nicotianae* population in South Africa and (v) determine the frequency and distribution of clonal genotypes amongst the different geographic regions.

MATERIALS AND METHODS

Fungal isolates

One hundred and five *P. nicotianae* isolates from citrus and tobacco hosts were collected in collaboration with the Research Institute for Tobacco and Cotton, Potgietersrus Tobacco Cooperative, Lowveld Tobacco Growers Association, Kutsaga Tobacco Research Institute and Outspan Citrus Center (Nelspruit, South Africa), as well as the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. All isolates have been deposited in the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), South Africa and the Cooperative Research Centre for Tropical Plant Protection, University of Queensland, Australia.

P. nicotianae isolates were baited from soil with *Citrus* leaf discs (Grimm & Alexander, 1973). Direct isolations from diseased host tissue were done on 3P selective medium (Tsao & Guy, 1977). *P. nicotianae* isolates were sub-cultured onto new plates containing selective media repeatedly until cultures were clean. For comparative purposes we also included four isolates (CMW 6917, CMW 7906, CMW 7907 and CMW 7908) from Zimbabwe.

DNA extraction

Cultures were grown in 200ml sterile 20% V8 broth (Erwin & Ribeiro, 1996). After 5 days at 25°C, mycelium was harvested and freeze-dried. Mycelium was ground in liquid nitrogen and DNA was extracted (Drenth *et al.*, 1993). DNA was quantified using a fluorometer and diluted to 18ng/ml for RAPD analysis. DNA samples were stored at –20 °C.

PCR amplification and sequencing of the ITS1 and ITS2 regions of rDNA

In order to determine if *P. nicotianae* isolates from tobacco and citrus belonged to the same species and to compare them with *P. nicotianae* from other studies (GenBank Accession number I41383) (Cooke & Duncan, 1997), DNA sequence was derived from the ITS1 and ITS2, as well as the 5.8S regions of the rDNAs from representative isolates. The rDNA repeat unit was amplified using the primers ITS 4 and ITS 6 (White *et al.*, 1990; Cooke & Duncan, 1997). Each PCR reaction contained 50ng template DNA, 1 mM of each primer, 100mM of each dNTP, 5μL 10 x PCR buffer, 1.5mM $MgCl_2$ and 2.5 Units Thermostable DNA Polymerase (Southern Cross Biotechnology (Pty.) Ltd., Cape Town, South Africa). The mixture was overlaid with 30μL of sterile mineral oil and thermal cycling was performed in an Omnigene thermocycler (Hybaid, Midlesex, United Kingdom) with an initial denaturation step of 2 min at 95°C. This was followed by 30 cycles of denaturation at 95°C (30 seconds), annealing at 55°C (30 seconds) and extension for 1 min at 72°C. A final extension step was performed at 72°C for 10 min.

The amplified rDNA fragments of 7 *P. nicotianae* isolates were sequenced. These included from 5 isolates (CMW 6913, CMW 6914, CMW 6915, CMW 6916 & CMW 6917) from tobacco and 2 isolates (CMW 6918 & CMW 6919) from citrus. The PCR products generated from these isolates were purified using a QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany). Sequencing reactions were performed with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, United Kingdom), and electrophoresed on an ABI PRISM 377 automated DNA sequencer.

Sequences were analysed using Sequence Navigator software (version 1.0.1; Perkin-Elmer Applied BioSystems, Inc., Foster City, California) and automatically aligned using Clustal V. The sequences were then aligned with all sequences from Clade I as described in Cooke *et al.* (2000) and percentage identity used as an indicator of species relatedness.

The identity of all other isolates as *P. nicotianae* was confirmed using the PCR-RFLP technique described by Cooke & Duncan (1997). For this purpose, the rDNA regions of all these isolates were amplified using the primers ITS 4 and ITS 6. PCR products were digested with *Msp* 1 and *Alu* 1 (Roche Diagnostics South Africa Pty., Ltd.) by adding 0.5µl

of either restriction enzymes *Msp* 1 and *Alu* 1 to 12 µl of each PCR product. These reaction mixtures were then incubated at 37°C for 1.5 hours. Ethidium bromide-stained digestion products were run on 2% high resolution agarose (Progen Industries Limited, Australia) and DNA restriction fragments were visualised using a UV transilluminator (302nm).

RAPD analysis

In order to determine the level of phenotypic diversity, each isolate was amplified with RAPD primers OPM-10, OPZ-04, OPZ-18, OPG-17 and OPH-07 (Operon Technologies) (Table 2). Amplification reactions were conducted in a 25μL volume consisting of 250µM for each dNTP, 25ng primer, 4mM $MgCl_2$, 60ng *P. nicotianae* DNA, 1.6 Units of *Tth* DNA polymerase (Biotech International, Australia), in PCR buffer (670mM Tris-HCl, 166mM $[NH_4]_2SO_4$, 4.5% Triton X-100 & 2mg/mL Gelatin). Amplification (FTS-320 Thermal sequencer) consisted of 39 cycles of 1 min denaturation at 94°C, 1 min annealing at 37°C, and 2 min extension at 72°C followed by 1 cycle of 1 min at 94°C, 1 min at 37°C, and 10 min at 72°C. Amplified RAPD DNA fragments were separated on 1.5% agarose RAPD TBE (0.89M Tris base, 0.89M Boric acid, 0.002M EDTA) gels, stained using ethidium bromide solution and visualised using an UV transilluminator.

Data analysis

The presence or absence of RAPD fragments was used to derive a multicharacter phenotype for each isolate. *P. nicotianae* isolates with the same overall multicharacter phenotype were considered clonal. Phenotypic diversity ($\hat{G}$) (Stoddard & Taylor, 1988) was calculated on the basis of the number of multilocus RAPD phenotypes within the *P. nicotianae* population studied. To compare levels of phenotypic diversity between the Lowveld and Northern Highveld regions, as well as tobacco and citrus *P. nicotianae* populations, diversity values of each population were corrected for sample size (McDonald *et al.*, 1994), to calculate the percentage maximum possible diversity obtained ($\hat{G}$/N%). The significance of differences in phenotypic diversity obtained with RAPD analyses for the Lowveld and Northern Highveld tobacco regions was calculated using a t-test (Stoddard & Taylor, 1988; Chen, Boeger &

McDonald, 1994). A t-test value was also calculated for differences between *P. nicotianae* tobacco and citrus populations, as well as, A1 and A2 mating type populations.

Mating type determination

In order to determine the frequency and distribution of mating types in *P. nicotianae* populations in South Africa, each isolate was paired with an A1 (ATCC 62653) and an A2 (ATCC 62654) test strain. All crosses were performed on 50% carrot agar and incubated at 24°C in the dark (Ribeiro, 1978). After a period of 14 to 21 days, the cultures were examined for the production of oospores. Isolates that produced oospores were considered to be of opposite mating type to that of the test strain.

Pathogenicity trial

The 4 isolates of *P. nicotianae* acquired for pathogenicity studies on tobacco and citrus were: 2 isolates (CMW 6921 and CMW 6923) from tobacco described as race 0 and 1 (Van Jaarsveld *et al.*, Chapter 2) and two isolates (CMW 7909 and CMW 7910) from citrus (Maseko, 1999). A single flue-cured tobacco cultivar, Coker 371-Gold and a single citrus cultivar, Rough Lemon were used to assess pathogenicity of the *P. nicotianae* isolates.

Eight-week-old-tobacco plants and 3-month-old citrus trees were used for these tests. The cambium of the test plants was removed with a cork borer (4mm diam.), 2 cm above ground level and a disc of agar, colonised with *P. nicotianae* was placed into the wound and sealed with parafilm. Ten tobacco plants and ten citrus plants were inoculated with each of the four *P. nicotianae* isolates. An equal number of control plants were inoculated with sterile disks of agar. A completely randomised block design was used in the trial and the entire trial was repeated once. In each case the tobacco plants were examined for lesion lengths after ten days while citrus plants were examined after 60 days. Re-isolations were also made from the resulting lesions on a medium containing Pimaricin (Tsao & Ocana, 1969) to verify that the inoculated pathogen was directly responsible for the lesion that developed. Data were statistically analysed for variance and differences among

isolates and means were tested for significance using Tukey's procedure (Steel & Torrie, 1980).

RESULTS

DNA based identification of P. nicotianae

The amplified rDNA sequences of all the *P. nicotianae* isolates had a length of approximately 852 base pairs (bp). The nucleotide differences in the analysed rDNA portions for the 5 isolates obtained from tobacco ranged from 2 to 25, whereas the 2 isolates from citrus differed from each other in 6 nucleotides. The differences between the tobacco and citrus isolates ranged from 5 to 19 nuceotides, with an overall identity of more than 99.99%. The South African *P. nicotianae* isolates also aligned with a high level of identity (99.97%) to the reference strains of *P. nicotianae* used by Cooke *et al.* (2000). Their rDNA sequences were also very closely related to those of other strains that were previously shown to be *P. nicotianae* (GenBank Accession number I41383) (Cooke & Duncan, 1997). The sequence analysis in combination with morphological observations confirmed that all isolates investigated were those of *P. nicotianae*. Digestion of the rDNA PCR products with restriction enzymes *Msp* I and *Alu* I produced identical RFLP- profiles for all of the 105 *P. nicotianae* isolates associated with citrus and tobacco (Fig. 2). These profiles were also similar to those of known *P. nicotianae* isolates (Accession nr. ATCC 62653 & ATCC 62654), confirming their morphological identification as *P. nicotianae*.

Genetic diversity

RAPD 's were used to analyse levels of phenotypic diversity within the South African *P. nicotianae* populations (Fig. 3). Thirty-nine polymorphic RAPD fragments revealed 79 different RAPD phenotypes among a total of 105 *P. nicotianae* isolates analysed (Tables 2 & 3). Lowveld *P. nicotianae* isolates were resolved into 54 and Northern Highveld isolates into 25 different RAPD phenotypes (Table 4). The 12 *P. nicotianae* isolates from citrus

hosts separated into 12 different RAPD phenotypes while the 89 *P. nicotianae* isolates from tobacco hosts separated into 67 different RAPD phenotypes (Table 4).

Regional diversity

Two out of the 79 different South African *P. nicotianae* RAPD phenotypes occurred in both the Northern Highveld and Lowveld. Within each region, unique RAPD phenotypes were found on farms (Table 3). The overall levels of phenotypic diversity for the different *P. nicotianae* populations were significantly higher for the Northern Highveld ($\hat{G}/N$= 93%) than the Lowveld ($\hat{G}/N$ = 45%) (Table 4).

Host diversity

Three out of 79 different RAPD phenotypes were found in both citrus and tobacco *P. nicotianae* populations. The overall levels of phenotypic diversity for the different *P. nicotianae* populations was significantly higher for the citrus population ($\hat{G}/N$= 100%) than the tobacco population ($\hat{G}/N$= 47%) (Table 4).

Mating type distribution

Of the 105 *P. nicotianae* isolates, 80 were of the A1 mating type, the remaining 25 represented the A2 mating type. Both mating types were found on both tobacco and citrus. On some farms (Carino farm 2 and Barberton), both mating types were found in the same field (Table 3). The overall levels of phenotypic diversity for the different *P. nicotianae* populations was significantly higher for the A2 population ($\hat{G}/N$= 100%) than the A1 population ($\hat{G}/N$= 43.9%) (Table 4).

Pathogenicity trial

Significant differences ($LSD_{citrus}0.05$ = 8.25, $LSD_{tobacco}0.05$ = 12.74) were found in lesion length associated with various *P. nicotianae* isolates inoculated onto citrus and tobacco

plants (Table 5). On tobacco plants of cultivar Coker 371-Gold, average lesion lengths associated with tobacco isolates CMW 6923 (68.20mm) and CMW 6921 (50.60mm) were significantly longer ($LSD_{tobacco}0.05 = 12.74$) than those associated with citrus isolates CMW 7909 (30.60mm) and CMW 7910 (31.70mm) (Table 5) (Fig. 4). All isolates were, however, pathogenic on tobacco plants and average lesion lengths differed significantly from the control (7.10mm) (Fig. 5). Average lesion lengths associated with the race 1 isolate CMW 6923 (68.20mm) was significantly greater on Coker 371-Gold than the race 0 isolate CMW 6921 (50.60mm), reflecting that this cultivar is resistant to race 0 of *P. nicotianae* (Table 5).

On the citrus cultivar, Rough Lemon, the average lesion length obtained with citrus isolates CMW 7909 (19.00 mm) and CMW 7910 (13.90 mm) did not differ significantly ($LSD_{citrus}0.05 = 8.25$) from each other or from the tobacco isolates CMW 6923 (17.50mm) and CMW 6921 (11.80mm) (Table 5) (Fig. 6). The tobacco isolates were therefore, as pathogenic on Rough Lemon as were the citrus isolates. Average lesion length obtained with all isolates on citrus plants differed significantly from the controls (3.40mm) (Fig. 7). *P. nicotianae* was consistently re-isolated from inoculated citrus and tobacco plants.

DISCUSSION

In this study, the causal agent of root rot on citrus and black shank on tobacco was confirmed as being *P. nicotianae* based on morphology and DNA sequence data for seven isolates and restriction digests of the amplified PCR product for 105 isolates. This result was not unexpected since *P. nicotianae* is a well-known pathogen of citrus and tobacco (Lucas 1975; Shew, 1991; Thompson *et al.*, 1995). It is, however, not the only *Phytophthora* species associated with tobacco and citrus (Shew *et al.*, 1999; Matheron, Wright & Porchas, 1998) and an important element of this study was to confirm the uniform occurrence on *P. nicotianae* in tobacco fields and citrus orchards.

Analysis of rDNA sequence data for *P. nicotianae* isolates showed high levels of identity to each other, regardless of the hosts from which isolates were collected (Cooke *et al.*, 2000). This finding is consistent with previous mtDNA RFLP studies (Panabiéres *et al.*, 1989; Förster, Oudemans & Coffey, 1990; Förster & Coffey, 1991) and isozyme data (Oudemans & Coffey, 1991), where no differences were found between *P. nicotianae*

isolates from different hosts. This is, however, in contrast, to the results of Colas *et al.* (1998) who reported that *P. nicotianae* isolates associated with black shank on tobacco may be distinguished from *P. nicotianae* isolates obtained from other hosts, based on mitochondrial and nuclear DNA RFLPs.

Using pathogenicity assays, we have shown that *P. nicotianae* isolates from citrus and tobacco hosts are able to cross infect their respective hosts. This supports the hypothesis that South African *P. nicotianae* isolates from citrus and tobacco belongs to the same pathogen population. *P. nicotianae* isolates from tobacco were as pathogenic as citrus isolates on the cultivar Rough Lemon. This is consistent with previous reports on the pathogenicity of *P. nicotianae* on a wide range of genera and plant families (Erwin & Ribeiro, 1996). Matheron & Matejka (1990), for example found that tomato (*Lycopersicon esculentum* Mill.) was highly susceptible to many *P. nicotianae* isolates including citrus isolates. *P. nicotianae* isolates from tobacco were, however, more pathogenic on tobacco than citrus isolates. Although our sample of isolates was relatively small, this result is consistent with previous reports that the highest degree of pathogenicity was shown by *P. nicotianae* isolates on their own hosts (Bonnet *et al.*, 1978; Matheron & Matejka, 1990). In future, growers will have to adapt cultural practices such as soil drainage and control of irrigation water to prevent cross infection of *P. nicotianae* isolates between tobacco and citrus fields (Collins & Hawks, 1993; Shoemaker, Shew & Main, 1999).

The A1 and A2 mating types were identified in *P. nicotianae* populations from the Northern Highveld and Lowveld. In some instances, both mating types of *P. nicotianae* occurred in single tobacco fields. These fields also harbored unique phenotypes in their *P. nicotianae* population. Results of this study further show that the South African *P. nicotianae* population had high levels of phenotypic diversity. This is similar to the results of population genetic studies on another *Phytophthora* species, such as *Phytophthora infestans* where high levels of phenotypic diversity and unique phenotypes were consistent with sexual reproduction (Goodwin *et al.*, 1992; Drenth *et al.*, 1994; Goodwin *et al.*, 1995; Mahuku *et al.*, 2000). Previous studies (Förster & Coffey, 1990) with *P. nicotianae* also indicate that sexual recombination occurs *in vitro*. Therefore, we hypothesise that the high level of phenotypic diversity found in *P. nicotianae* populations in South Africa is due to occasional sexual recombination in these populations. However, the occurrence of sexual

reproduction cannot be tested using phenotypic markers and a separate study utilising larger samples size and co-dominant genetic markers is needed to rigorously test this hypothesis.

A sexually propagating *P. nicotianae* population is a constant threat to tobacco cultivation in South Africa. With each sexual recombination, new combinations of virulence genes may arise with the ability to overcome combinations of resistance genes in host plants (McDonald & McDermott, 1993). This may result in more aggressive and virulent *P. nicotianae* populations. During sexual reproduction, oospores are formed that survive in the soil between tobacco seasons (Chern & Ko, 1994; Masuka & Namichila, 1996), thereby contributing to the spread of the disease as well as increasing the amount of inoculum present in the field.

This study provides interesting and new insight into the population structure of *P. nicotianae* in South Africa. RAPD analysis revealed 79 RAPD phenotypes among 105 isolates of *P. nicotianae*. These phenotypic groups were not limited to a specific tobacco region and two phenotypic groups occurred in both the Northern Highveld and Lowveld region. The high levels of phenotypic diversity found in the Northern Highveld and Lowveld *P. nicotianae* populations warrants careful selection of *P. nicotianae* isolates in breeding trials for black shank resistance. *P. nicotianae* isolates should be aggressive and representative of the prominent phenotypic groups in an area or site where newly bred cultivars will be deployed.

Thirty-five of the 105 *P. nicotianae* isolates included in this study were clonal. Identical *P. nicotianae* clones were isolated from both citrus and tobacco hosts. This is consistent with the ability of South African *P. nicotianae* isolates to infect both citrus and tobacco hosts. Our results support those of Drenth *et al.* (1993) who reported that clonal genotypes of *P. infestans* also occurred on more than one host.

Clonal *P. nicotianae* isolates were not limited to a single site and occurred in Northern Highveld and Lowveld area. For example, one *P. nicotianae* clone was found in Potgietersrus, Naboomspruit (Northern Highveld) and Carino (Lowveld). The practice of distributing tobacco seedlings from one area to another may have contributed to the spread of clonal *P. nicotianae* isolates since infected seedlings have been the major agents of black shank spread in Australia and the USA (Tisdale & Kelley, 1926; Lucas, 1975; O'Brien &

Davis, 1981). Our results support the view that effort must be made to limit the distribution of infected tobacco planting stock in South Africa.

Results of this study have provided valuable information on the population genetic structure of South African *P. nicotianae* populations. Knowledge of the phenotypic groups, the distribution of clones and mating types will be useful to breeders in their efforts to develop and deploy new cultivars. Knowledge regarding the ability of South African *P. nicotianae* isolates to infect both citrus and tobacco will also enable growers to adjust management practices to reduce the impact of this pathogen.

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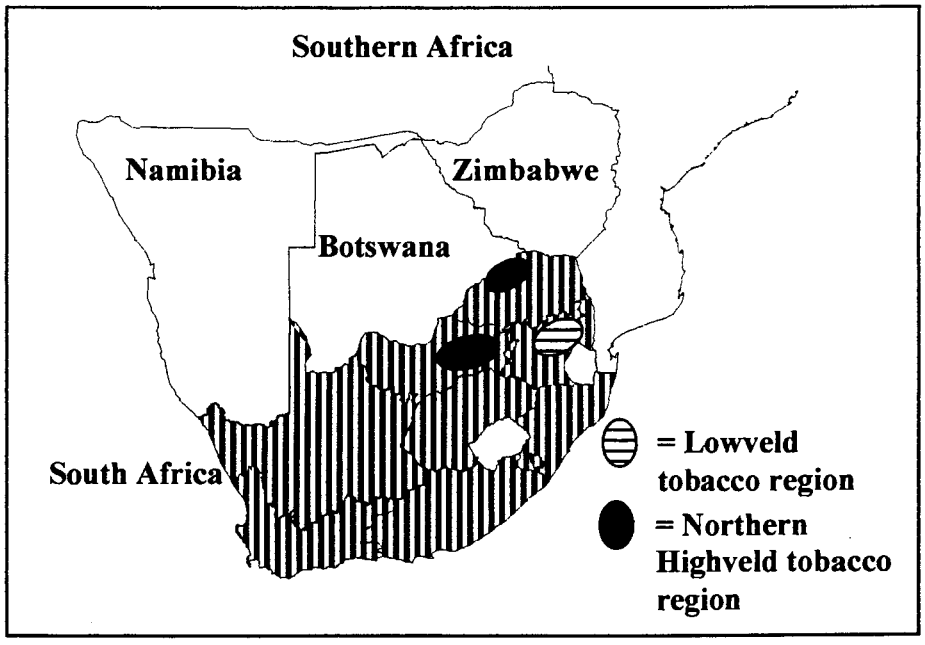
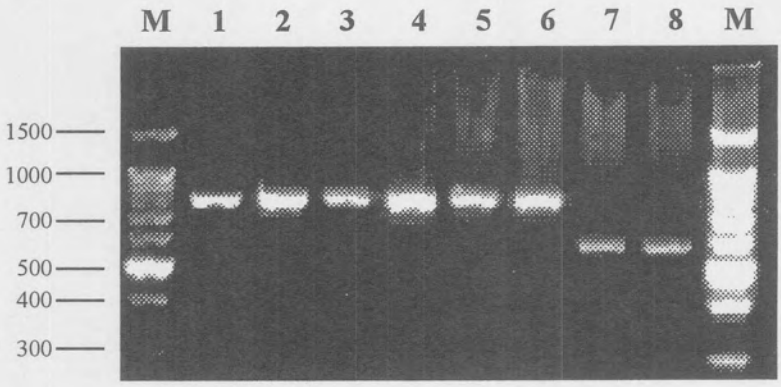


Fig. 1 Tobacco producing regions in South Africa.

Fig. 2 Agarose gel (3%) of PCR amplification products of the ITS1/5.8S/ITS2 region of the RNA gene repeat of 2 *Phytophthora* spp. (primers ITS 4 & ITS 6) digested with *Alu* I (**A**) and digested with *Msp* I (**B**), 100 bp ladder in the first and last lane; 1 an 2 *P. nicotianae* from citrus host, A2 mating type; 3 and 4 *P. nicotianae* from tobacco host, A2 mating type; 5 and 6 *P. nicotianae* from tobacco host, A1 mating type; 7 and 8 *P. cinnamomi*, A2 mating type.

A



B

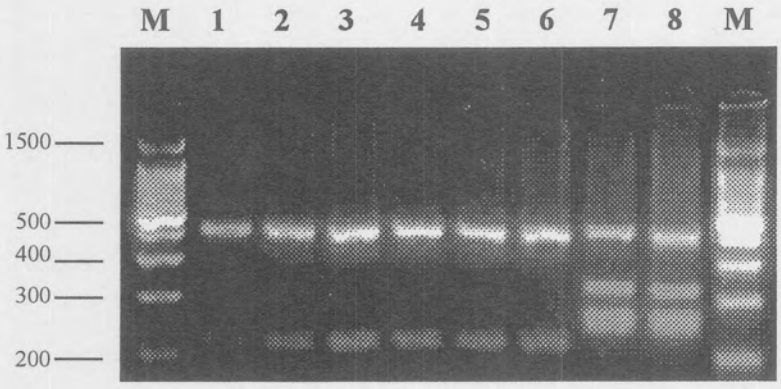
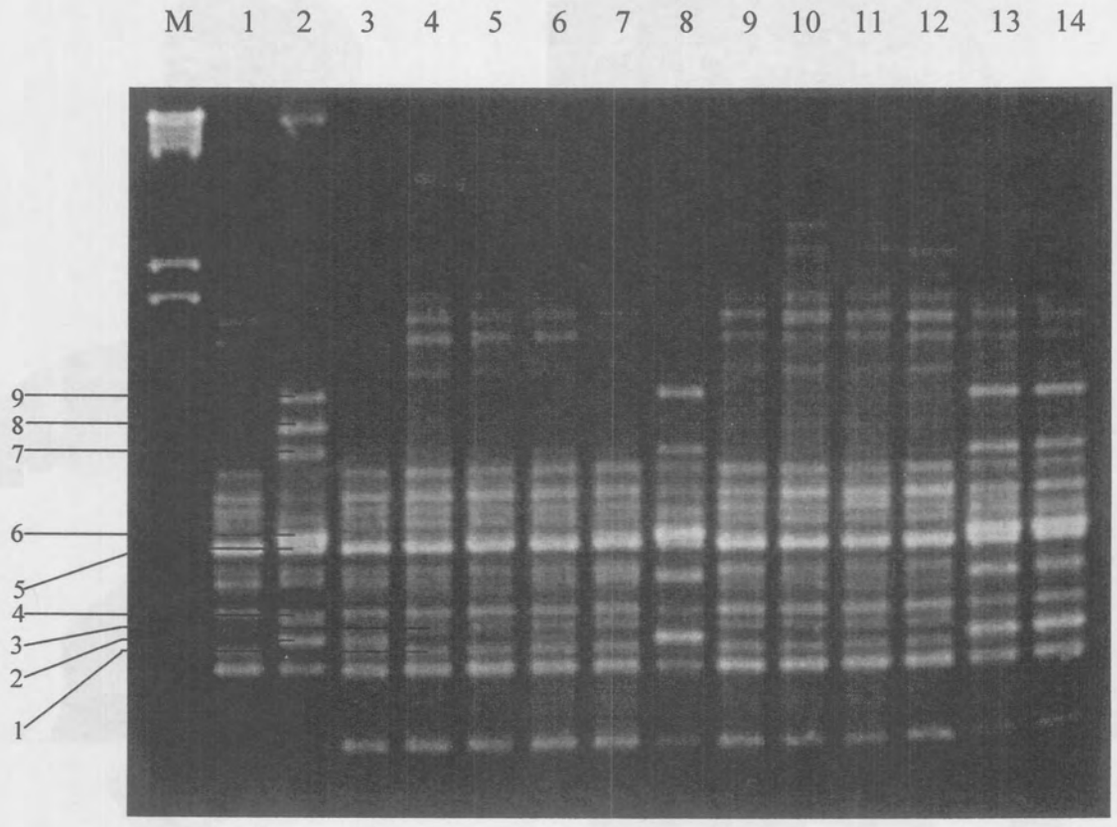


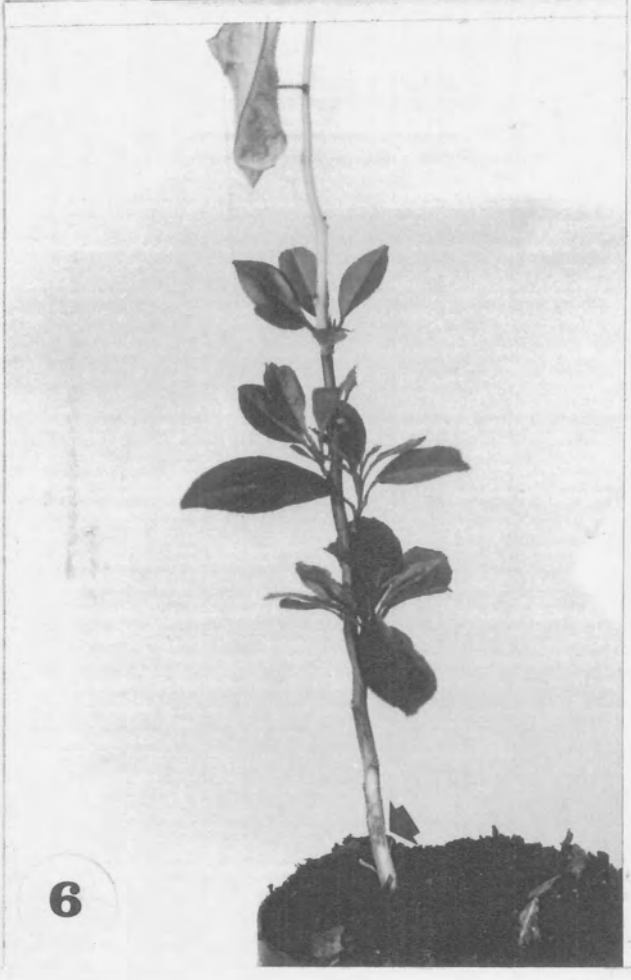
Fig. 3 RAPD profiles generated by OPM10 for selected *Phytophthora nicotianae* isolates. Lane M corresponds to molecular size markers (100-bp markers). Lanes 1 and 2 are *P. nicotianae* isolates from Schagen while lanes 3 and 4 are isolates from Carino farm 1. Lanes 5 to 13 are *P. nicotianae* isolates from Carino farm 2 and lane 14 is an isolate from Barberton.



Figs. 4-7 Tobacco and citrus plants inoculated with *Phytophthora nicotianae* isolates and sterile agar. **Fig. 4** A tobacco plant exhibiting wilting symptoms and a black discoloration (arrow) on the stem after inoculation with a *P. nicotianae* isolate from citrus. **Fig. 5** A healthy control tobacco plant inoculated with sterile agar. **Fig. 6** A Citrus tree exhibiting wilting symptoms and a brown discoloration (arrow) on the stem after inoculation with a *P. nicotianae* isolate from tobacco. **Fig. 7** A healthy control citrus tree inoculated with sterile agar.

4

5



6

7

Table 1. *Phytophthora nicotianae* isolates used in this study

Isolate collection	Site	Sample size	Host
Area			
Northern Highveld	Potgietersrus	6	tobacco
	Naboomspruit	2	tobacco
	Kroondal	13	tobacco
	Rustenburg	2	tobacco
	Levubu	1	tobacco
	Letaba	2	citrus
	Total	26	
Lowveld	Carino farm 1	7	tobacco
	Carino farm 2	40	tobacco
	Schagen	5	tobacco
	Barberton	13	tobacco
	Nelspruit	7	citrus
	Komatipoort	3	citrus
	Total	75	
Zimbabwe	Kutsaga	4	tobacco
	Total	4	
Total number of isolates		105	

Table 2. RAPD primers used and number of amplified polymorphic and monomorphic observed fragments observed among the *P. nicotianae* isolates

Oligonucleotide primers [1]	Primer sequence	Observed fragments	
		Polymorphic	Monomorphic
OPM-10	5'-TCTGGCGCAC-3'	9	10
OPZ-18	5'-AGGGTCTGTG-3'	7	6
OPG-17	5'-ACGACCGACA-3'	10	5
OPZ-04	5'-AGGCTGTGCT-3'	6	9
OPH-07	5'-CTGCATCGTG-3'	7	6
Total		39	36

[1] Oligonucleotide primer obtained from Operon ®

Table 3. *P. nicotianae* mating types and RAPD phenotypes found in geographical areas

Area	Site	Sample size	Mating type		Number of RAPD distinct phenotypes	Unique phenotypes in site
			A1	A2		
Northern Highveld	Potgietersrus	6	6	0	6	5
	Naboomspruit	2	2	0	2	1
	Kroondal	13	2	11	13	13
	Rustenburg	2	2	0	2	2
	Levubu	1	1	0	1	1
	Letaba	2	0	2	2	2
	Total	26	13	13	25	
Lowveld	Carino farm 1	7	7	0	7	5
	Carino farm 2	40	38	2	30	22
	Schagen	5	5	0	5	4
	Barberton	13	12	1	12	7
	Nelspruit	7	2	5	7	6
	Komatipoort	3	0	3	3	2
	Total	75	64	11	54	
Zimbabwe	Kutsaga	4	3	1	2	2
Total		105	80	25	79	

Table 4. Phenotypic diversity in *P. nicotianae* populations

Population differentiation		Sample size	Distinct RAPD phenotypes	Phenotypic diversity Ĝ	Ĝ /N %	t-test	Probability
Region							
	Northern Highveld	26	25	24.1	93%	1.88	0.025 *1
	Lowveld	75	54	33.7	45%		
Host	Tobacco	89	67	41.5	47%	3.01	0.0025*1
	Citrus	12	12	12.0	100%		
Mating type	A1	80	57	35.2	43.90%	2.92	0.0025*2
	A2	25	25	25	100%		

*Populations do not differ significantly according to t-test (Chen *et al.*, 1994)

[1] Significance $P < 0.05$, degrees of freedom 99

[2] Significance $P < 0.05$, degrees of freedom 103

Table 5. Comparison of pathogenicity of *P. nicotianae* isolates

			Lesion lengths (mm)[1]	
P. nicotianae isolates	Host	Race	Tobacco Coker 371-Gold	Citrus Rough Lemon
CMW 6923	Tobacco	1	68.2 d[2]	17.50 x[3]
CMW 6921	Tobacco	0	50.6 c	11.80 x
CMW 7909	Citrus	-	30.6 b	19.00 x
CMW 7910	Citrus	-	31.7 b	13.90 x

[1] Each value represents the mean of 10 replicates

[2] Within each column, mean values followed by the same letter are not significantly different $LSD_{tobacco}$ (0.05) = 12.74

[3] Within each column, mean values followed by the same letter are not significantly different LSD_{citrus} (0.05) = 8.25

SUMMARY

As the causative agent of black shank, *Phytophthora nicotianae* is a serious threat to tobacco cultivation in South Africa. Research presented in this dissertation describes pathogenicity studies and control measures for *P. nicotianae* on tobacco. Special attention is given to the population structure of *P. nicotianae* in South Africa. The implications of these genetic studies in breeding and selection programs against *P. nicotianae* were also evaluated.

The first chapter of this dissertation represents a literature review on black shank and available control measures for *P. nicotianae* on tobacco. The mechanisms of pathogenicity and the life cycle of *P. nicotianae* are also treated in detail. Special reference is made to the maintenance of genetic diversity in *Phytophthora* species and particularly *P. nicotianae*. This literature review also highlights the fact that very few studies have been conducted to determine the genetic structure of *P. nicotianae* populations.

The success of South African breeding programs for tobacco cultivars with *P. nicotianae* resistance is to some degree dependent on the selection of isolates with high levels of aggressiveness. The research presented in chapter two provides information on cultivar resistance and selection of *P. nicotianae* isolates for future breeding programs. Significant differences in levels of aggressiveness were found between *P. nicotianae* isolates. Furthermore, race 0 and 1 of *P. nicotianae* occurred in most of the tobacco growing regions in South Africa. Selected Race 0 and 1 isolates were thus used to evaluate black shank resistance of 11 commercially planted tobacco cultivars. Commercially planted cultivars differed significantly in their resistance to race 0 and 1. Cultivars LK33/60 and OD1 were highly resistant to race 0 but susceptible to race 1 while cultivars Vuma/3/46 and LK3/46 were highly resistant to both race 0 and 1.

Chapter three reports on the use of metalaxyl treatments combined with resistance in tobacco cultivars for control of *P. nicotianae*. One hundred and thirty two isolates of *P. nicotianae* were screened for sensitivity to metalaxyl. *P. nicotianae* isolates from most tobacco farms were metalaxyl sensitive. The results further indicated that the use of metalaxyl in combination with moderately resistant cultivars effectively reduced black shank in the field. The outcome of this study provided useful information for the

implementation of an economically viable combination of disease resistance and metalaxyl as the basis for a *P. nicotianae* management program in South Africa.

Chapter four of this dissertation deals with the development of a rapid seedling-based screening technique to assay tobacco for resistance to *P. nicotianae*. This technique was validated by comparing it to a stem inoculation technique commonly used on adult plants. A strong positive correlation was found between results of the seedling assay and adult plant trials for all isolates and cultivars tested. *P. nicotianae* isolates could also be characterized as race 0 or 1 using both stem inoculation and the rapid seedling assay. The ability to screen large numbers of tobacco plants rapidly at the seedling stage allows for the testing of large germplasm resources in a systematic manner and under standard conditions. This may help in the timely development and release of more black shank resistant cultivars.

In chapter five, a population study on *P. nicotianae* in South Africa is presented. One hundred and five *P. nicotianae* isolates were collected from the Northern Highveld and Lowveld regions, as well as from both citrus and tobacco hosts in South Africa. Levels of phenotypic diversity were determined in populations of *P. nicotianae* using RAPD markers. Among the 105 *P. nicotianae* isolates analysed 79 different RAPD phenotypes were found, where 35 of the isolates were found to be clonal. The high number of RAPD phenotypes (79) in relation to the sample size (105), the presence of both the A1 and A2 mating type and high levels of phenotypic diversity in the *P. nicotianae* population indicate a sexually outcrossing *P. nicotianae* population in South Africa. This sexual outcrossing may mean that *P. nicotianae* is likely to remain a constant threat to tobacco and citrus cultivation, since new genotypes with the potential to overcome resistance genes in commercial cultivars are likely to emerge.

All chapters of this dissertation deal with some aspects of black shank control and breeding for resistance to *P. nicotianae*. This dissertation provides new knowledge on variation in levels of aggressiveness, race distribution and the development of metalaxyl resistance in the South African *P. nicotianae* populations. This also represents the first study on the genetic diversity of *P. nicotianae* populations in South Africa. The results presented here not only show the possible occurrence of sexual reproduction, but also indicate the presence of clones and discreet phenotypic groups of *P. nicotianae*. This information will be

applied in future tobacco breeding programs to select breeding lines with resistance against a number of specific *P. nicotianae* races and phenotypic groups.

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