STUDIES ON GUAVA WILT DISEASE

Nigel Mark Grech

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University of KwaZulu-Natal

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THESIS SUMMARY

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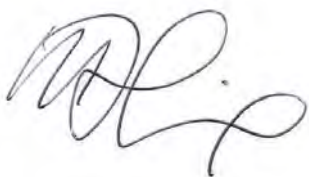
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TABLE OF CONTENTS

CHAPTER 11: LABORATORY SENSITIVITY OF *NALANTHAMALA PSIDII* TO METHYL BROMIDE, AND FIELD EVALUATION OF THE FUMIGANT FOR THE ERADICATION OF *N. PSIDII* IN CONTAMINATED ORCHARD SITES

CHAPTER 12: EVALUATION OF CHEMOTHERAPEUTIC AND BIOLOGICAL AGENTS ALONE AND IN COMBINATION WITH METHYL BROMIDE SOIL FUMIGATION FOR EFFICACY IN GUAVA WILT DISEASE MANAGEMENT

CHAPTER 1: INTRODUCTION, LITERATURE REVIEW AND GLOBAL DISTRIBUTION OF GUAVA WILT DISEASE

1.1 Guava

The common guava (*Psidium guajava L.*) is classified within the Myrtle family and is the most important commercially cultivated fruiting genus (Popenpoe, 1948) within a family of over 140 plant genera and 3000 species (Ellshoff, *et al*., 1995). The *Myrtaceae* also includes such notable genera such as *Eucalyptus* (Gum trees), *Eugenia* (including Cloves and Rose apples), *Feijoa*, *Pimenta* (Pimento), *Callistemon* spp. (Bottlebrush), *Leptospernum* spp (Australian teabush), and a large variety of lesser known plants. The Myrtle family is ancient and primarily distributed on three Gondwanan derived continents, namely Australia, South America and Africa (Sytsma, *et al*., 2004). Evolving in the warm, moist tropics, plants in this family have geographically spread and climatically diversified and in so doing, have become significantly more specialized such as the species found in the arid regions of Western Australia. Native species within the Myrtle family are also found in Southern Europe (1 genus), Asia, and are confined to a few Genera in the southern regions of North America (Berry, 1915).

The genus *Psidium* contains approximately 150 species of which 30 are considered edible (Kwee and Chong, 1990) and of which there are twelve cultivated species that are of commercial importance: *P. guajava* (common guava), *P. montanum* (mountain guava), *P. littorale* (yellow/strawberry guava), *P. freidrichstalianum* (Costa Rican or wild guava), *P. cattleianum* (strawberry guava), *P. sellowiana* (pineapple guava), *P. guinense* (Brazilian guava), *P. cinereum* (araca do Campo), *P. firmum* (araca), *P. salutare* (araca-rasteiro), *P. rufum* (araca roxo) and *P.*

acutangulum (para guava). All guava species are indigenous to the tropics of America (Morton, 1987, Kwee and Chang, 1990). The fruit were first reported growing in that region as early as 1526 by Gonzalo Hernandez de Olveido, who observed their use by the Aztecs, who called the fruit "xalxocotl" meaning sand plum, a probable reference to the high number of stone cells in the fruit flesh. Olveido was largely responsible for the fruits' introduction into the West Indies and later the Philippines from where it was disseminated across the tropics of Asia. In 1754, Ehret, Trew and Haid published >*Plantae Selectae Quarum Imagines ad Exemplaria Naturalia Londini in Hortis Curiosorum Nutrita Manu Artificiosa Doctaque Pinxit* ' (Fig.1.1) which approximately translates to read, >"A Collection of Selected Plant Images to Model Nature in London and to Encourage Curiosity by Learned Hand Paintings"=. This was the first botanical description of guava to appear in Europe. In this classic botanical work, the authors described and painted plants they encountered in their travels through Central and South America. The guava featured prominently in this work and detailed water color depictions of the plant are found in the book (Fig. 1.2). Indeed, upon closer examination of the water color descriptions of guava featured in the book, the authors inadvertently also illustrated what we now know to be anthracnose symptoms on the fruit (Fig 1.3) caused by *Colletotrichum gloeosporioides* (Penzig.), the anamorphic state of *Glomerella cingulata* (Stonemam.) Spauld. and Schrenk.

According to Dweck (2003), the common guava is known outside the tropical regions of the Americas by a wide array of names around the world such as: yellow guava and apple guava. In the Philippines it is known as: bayabas, tayabas, guayabas (Tagalog); bayabas, guayabas (Llokano). In Malaysia the local names are: jambu burung, jambu padang, jambu berasu, jambu

buyawas, jambu Portugal, jambu batu, jambu biji, (Bahasa Malaysia). In West Africa some common names for the guava are: gwaabaa (Hausa); woba (Efik); ugwoba (Igbo); guafa (Yoruba); gua, aduaba; oguawa, eguaba, gouwa (Ewe).

In India, the guava has a wide variety of names reflective of the great diversity of dialects found in that country, such as, perala (Sans); lal sufrium (Hind); lal peyara and goachi-phal (Bem); perala (Bom); Jama; Jam-pandu (Tel); paera (Kom); zetton and jamphal (Sind); peru and jamba (Mah); jamrukh (Guj); madhuria (Assam); amuk (Nepal); amrud (Arab and Persian); amrut (Punjabi); malakabeng (Burmese). In Central America, guayava and guayabo (Spanish); goyave (French); guyaba (Dutch) and guajaba (Haitian) from which the English name "guava" is probably derived (Nakasone, 1998). These names from all over the world reflect the broad guava diaspora that has occurred since the fruit was first discovered by Europeans hundreds of years ago. In fact guavas are now cultivated on all continents (except Antarctica) and are truly pantropic. Guavas are found throughout the tropical and subtropical regions of the world with a general climatic limit of distribution between the latitudes of 35^B north and 35^B south. The common guava is limited to elevations below 2000 m that are frost free. In many countries the guava has rapidly become successfully naturalized, being largely spread by birds (Somarriba and Somarriba, 1985a). In many countries the common guava (and other closely related species) have become aggressive invaders of pastures as in Natal, South Africa (Baijnath *et al*., 1982), Costa Rica (Somarriba and Somarriba, 1985b, 1987), Mauritius (Macdonald *et al*., 2003) as well as Fiji, and Hawaii (Huenneke and Vitousek, 1990) where in some areas it is a declared a noxious weed, requiring intensive management (Santos *et al*., 1989). Guavas are grown for the fresh, dried fruit, juice, puree, powder

or canned markets (Chan, 1983). Non-conventional processed products include seed oils (Pathak and Dixit, 1984; Saha and Nagar, 1984), fodder (Hugar *et al*., 1991), pectin products (Duke and Du Cellier, 1993), insect repellents (Sharby, 1989), wine (Dhawan *et al*., 1983) and tea (Grande and Wondergem, 1985). The inherent versatility of the guava has resulted in the fruit gaining greater grower and consumer attention in the more temperate climes of Europe and North America (Proctor, 1990). Indeed this versatility has also resulted in many problems experienced by the grower in trying to produce a crop for several divergent uses (Ito *et al*., 1990). Besides these conventional uses, guavas have an important role in ethnopharmacology. The effects of various guava preparations used in traditional medicines are well documented (Burkhill *et al.,* 1985) and until recently much of the pharmacology of these observed effects were unknown (Meckes *et al.,* 1996). Many cultures around the world use certain guava tissues in various formulations for the treatment of ailments such as gout (Duke and Vasquez, 1994), toothache (Burkhill *et al*., 1997), diarrhea (Van Wyk *et al*., 1997) as well as being used as an astringent and as a wound dressing (Grande and Wondergem, 1985; Lutterodt, 1989, Duke and Vasquez 1994). More recently products containing ground, dried guava leaves, are being marketed as a cure for hangovers (Anonymous, 2015).

1.2   Guava Production

Approximately 300,000 ha of guava are under commercial cultivation worldwide (Grech, 1988; Anonymous, 2010), excluding subsistence guava farming, which would greatly increase these area estimates. Commercial production is reported in 58 countries (Knight, 1980), with an estimated global production of 500,000 MT in 1988 (Jagtiani *et al*., 1988) and increasing to 4 million tons

by 2004 (Pommer and Murakami, 2009). India has the largest reported area under guava cultivation (Singh *et al.,* 2007; Vigneshwara, 2013) at approximately 210,000 ha, followed by Pakistan at 60,000 ha (Anonymous, 2008 and 2010; Hassan, 2012), Mexico (15,000 ha), Brazil (20,000 ha), Colombia and Egypt each at 3000 ha (Adsule and Kadam, 1995; Anonymous, 2010). These figures do not include production from wild guavas. Indeed in some developing countries the guava tree is of great importance as a source of fuelwood (Somarriba and Beer, 1985; Somarriba and Somarriba 1985a, 1985b, 1987; Roy and Gill, 1990) and the tree is often coppiced for managed renewal of this fuel resource. The fruit has one of the highest per unit ascorbic acid levels (above 200mg/100g fruit (Waddington and Cist, 1942) and has high consumer level of appeal (Proctor, 1990). Indeed in the South African juice market, guava juices out sells all other fruit juices (Guava Growers Association, personal communication, 1988). Guava fruit has also been found to be a fair source of calcium, phosphorus and vitamin A.

The guava industries of Southern Africa are primarily located in the Cape winter rainfall region, the Mpumalanga Lowveld, the Limpopo Province and the Swaziland and Mozambique Lowveld regions, totaling approximately 1400 hectares in 1982, declining to approximately 1000 ha by 1985 and 650 ha by 1992 [of which approximately 500 ha were in the guava wilt disease (GWD) free Western Cape regions (Anonymous, 1983; Grech 1985; Grech and Rijkenberg, 1987; De Villiers and Joubert, 2004)]. By 2006 there had been some recovery in the area planted to guava in the Limpopo and Mpumalanga Provinces to approximately 600 ha primarily due to the introduction of the GWD tolerant guava selections, TS-G1 and TS-G2 (Schoeman *et al*., 1997; Schoeman *et al*., 2012; Severn-Ellis *et al*., 2012). However by 2009 a renewed GWD outbreak was reported, but in this epiphytotic, TS-G1 and TS-G2 were observed to be affected by this malady and the ensuing orchard destruction once again reduced the planted hectarage in these provinces and led to widespread abandonment of guava cultivation in favor of other crops (Schoeman and Labuschagne, 2014).

1.3   Guava Wilting Disease

Historically, guavas globally have been looked upon as a low maintenance tree crop capable of growing in poor soils and are widely thought of as being a secondary crop in commercial fruit growing operations. Prior to the mid-eighties the only diseases of any consequence to the South Africa grower were of the fruit (Matthee, 1981). The major diseases of guava are fungal in origin, with few reports of bacterial or viral induced maladies on the crop. Indeed, over the last several decades, only one report of a viral disorder of the crop has been found by the author (Sidek and Kwee, 1990) as well as one report of a bacterial disease caused by *Erwinia psidii* (Rezende *et al*., 2008).Wilting diseases of guava can inflict severe losses in an industry, as has been the case in India (Ray, 2002). South African guava farmers were fortunate that for many

years, devastating wilting diseases were not present in the main production areas. However, the situation for guava growers in Southern Africa catastrophically changed in the early nineteen eighties, as a new lethal disease, guava wilt disease (GWD) became established in parts of the north eastern and eastern regions of the sub-tropical Lowveld of South Africa (Grech, 1983). By the late eighties, the disease had affected all the major growing regions (outside of the Cape Province) and had resulted in an approximate yield loss in the national industry of approximately forty percent (Grech, 1988). Legislation was promulgated in 1985 as a result of the appearance of the disease in the sub-tropical regions of South Africa, restricting the movement of propagation material from the northerly infected regions to the southerly (Cape Province) disease free regions[1]. In the field, affected trees exhibited a wide range of symptoms that showed seasonal variation. Without exception, once diseased, trees failed to recover and usually died within six months. The prognosis for guava growers was worsened by the fact that the industry relied on one dual purpose, commercial cultivar, a cultivar that proved to be very susceptible to this malady. By the early 1990's the tree loss due to the GWD approached 70% in the Mpumalanga province. This study also identified other regions of the world where GWD occurs. Several visits were made to the Republic of China, Taiwan to study GWD. In addition, several other countries were visited to investigate the possible incidence of GWD in those countries (Philippines, Malaysia, Thailand, Indonesia, China, Mexico, Costa Rica, Panama, Colombia, Brazil, Peru, Ecuador and Honduras). Reports of GWD were investigated primarily in Southern Africa but also in several countries that had not previously reported (or confirmed) the presence of GWD. Suspected cases of GWD were studied by recording the symptoms, collecting guava tissue isolations and attempting to recover

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[1] www.guavaproducers.co.za

the causal organism, as well as testing the pathogenicity of any novel isolates, using the methodologies described in Chapter 2.
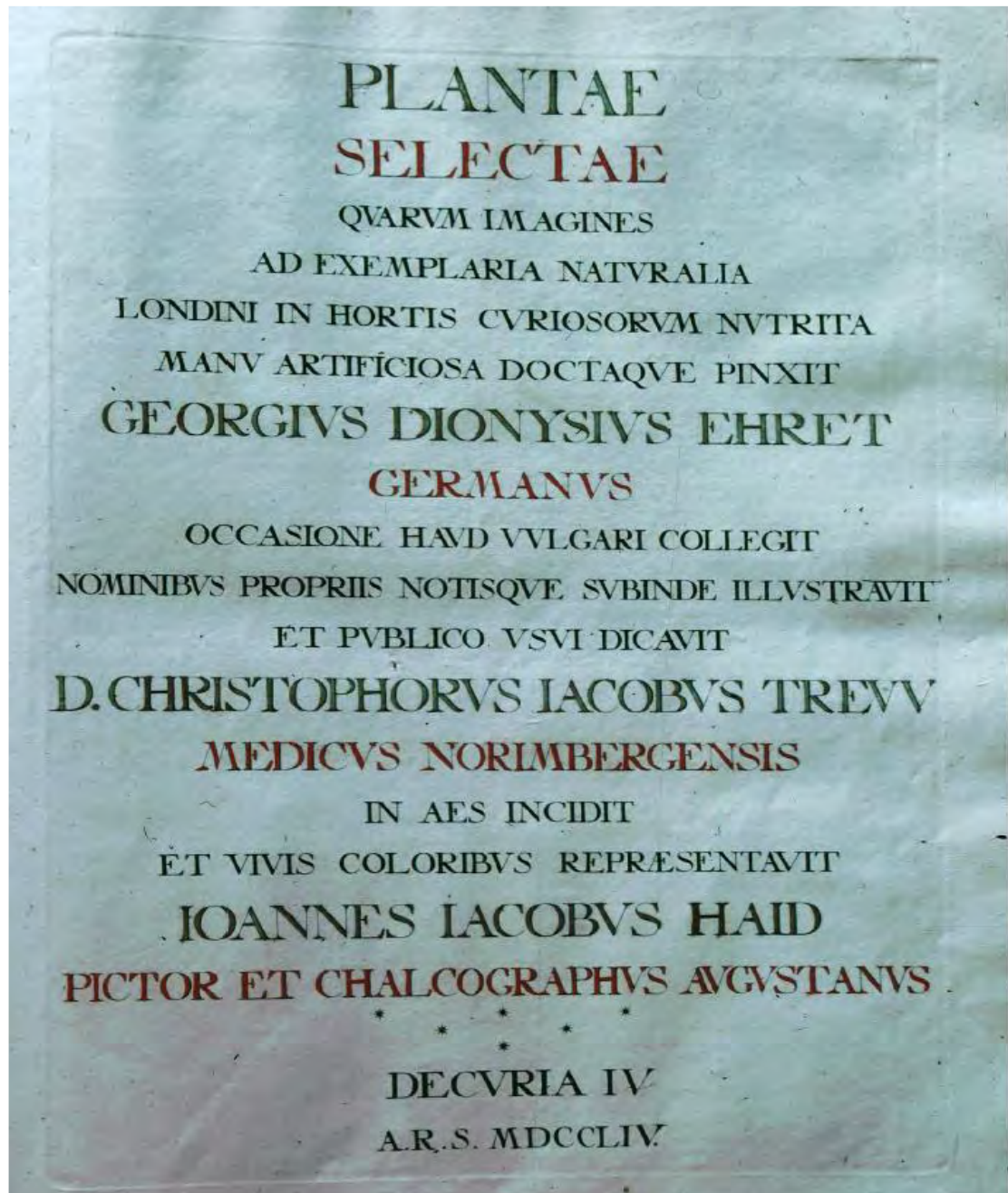
Fig.1.1 *Plantae Selectae Quarum Imagines ad Naturalia Londini in Hortis Curiosorum Naturita Manu Artificiosa Doctaque Pinxit.* Published in 1754.

Fig. 1.2. The first recorded pictorial description of *Psidium guajava* to appear in print in 1754. The translated text reads: *Guava leaf branches four sided, fruit oblong, flesh red. Figure 1. Branch with anterior flowers, a. Rear view of a single flower. Figure2. Branch with fruit, b. Prepared transverse fruit section, c. Seeds.*
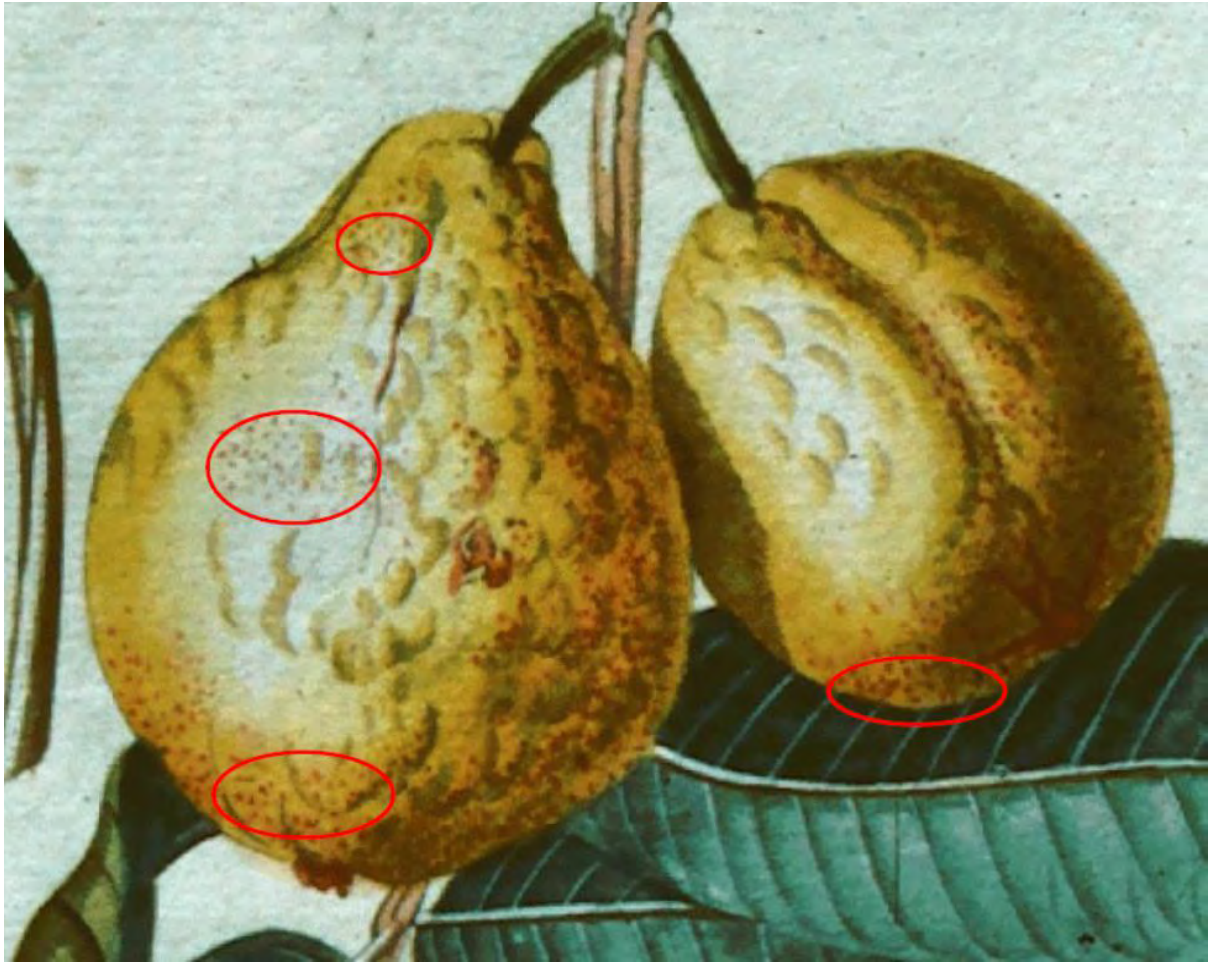
Fig. 1.3. A close up of the fruit from Figure 1.2. The red circled areas are suspected anthracnose fruit spots caused by *Colletotrichum gloeosporioides*, a common fruit pathogen of guava in the American tropics and subtropics.

Figure 1.4. Known global distribution of guava wilt disease.

Wilting diseases of guava have been reported from many areas of the world where guava is cultivated (Misra, 2007). Reports from India as early as 1945 (Hayes), 1947 (Das and Rai) and 1955 (Chattopadhyay and Gupta), identified the cause as *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans. Wilting maladies of guava have been reported in the USA (Alfieri *et al.*, 1984), Mexico and Honduras (Grech, 1994), Australia (Nakasone, 1998), Pakistan (Hamiduzzaman *et al*., 1999), Taiwan, Republic of China (Leu and Kao, 1979), South Africa (Grech, 1985), Malaysia (Schroers, *et al*., 2005; Ploetz, 2007), The Philippines (Quimio *et al.,*

1984, Barnett, R., pers. comm[1]., 2001) and Thailand (Athipunyakom and Manoch 1998; Athipunyakom and Luangsaard 2008). Currently the known global distribution of GWD is outlined in Figure 1.4.

Guava wilting disease (GWD) is the most destructive disease of guava. GWD has been positively identified from Taiwan, where it is known locally as "Li-ku-bin" (Kurosawa, 1926), South Africa, Mozambique and Swaziland (Grech, 1983). By 1987, approximately one million trees had died of this disease in those countries (Grech and Rijkenberg, 1987; Grech, 1987).

Schoeman (unpublished in Schroers *et al*., 2005) reported a wilting disease of guava from Malaysia. GWD has been confirmed in Thailand (Athipunyakom and Manoch, 1998) and identified at that time as *Acremonium psidii*. Recently, the identification of the fungus was confirmed in both Malaysia and Thailand (Athipunyakom and Luangsaard, 2008) as *N. psidii*. In the Philippines an *Acremonium* wilt of guava was reported by Quimio *et al.,* (1984). The cause of the GWD reported in India, (Chattopadhyay and Gupta, 1955; Pandey and Dwivedi, 1985) was ascribed to the fungus *Fusarium oxysporum* Schlecht f.sp. *psidii* (Edward, 1959). The symptomatology and epidemiology of GWD as described in India are very similar to those described in South Africa and Taiwan (Grech, 1987; Dwivedi *et al*., 1988). However, to date the GWD pathogen reported in India has not been confirmed as being the same as the pathogen

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[1] Roberto Barnett. Senior Field Agronomist Chiquita Brands. Bo Land, Davao City, Mindanao, Philippines, 8000

in Taiwan or Southern Africa, although Misra (2007) suggested that *Gliocladium roseum* Bainer as being the most potent wilting pathogen of guava isolated to date in India.

1.4   The Identity of the Pathogen Causing Guava Wilting Disease

The taxonomic status of the fungi causing GWD in the Republic of China (Taiwan) and the Republic of South Africa appeared to be taxonomically similar (Grech, 1988). However, until recently (Schroers *et al.,* 2003; 2005), the taxonomic status of the fungus was unclear. Initially the GWD inducing fungus in South Africa was identified as *Septofusidium elegantulum* (Pidlopl.) W. Gams (Roux, 1982, pers. comm.[2]), a *Paecilomyces* sp. (Grech, 1983), a *Gliocladium* sp Corda (Grech, 1983), *Gliocladium vermoesenii* (Biourge) Thom and G*liocladium catenulatum* Gilman and Abbott (Brady, 1983, pers. comm.[3]), *Gliocladium vermoesenii* (Sutton, B.C. 1983, pers. comm.[4]), *Nalanthamala madreeya* Subram (Gams, W., pers. comm., 1985[5]), a *Gliocladium* sp. (Menge 1994, pers. comm.[6]) and *Penicillium* Biourge [=*Gliocladium vermoesenii* (Biourge) Thom., (Schoeman *et al.,* 1997, Vos and Schoeman 1998, Geldenhuis *et al.,* 2000)]. The GWD pathogen has been previously compared to the persimmon wilt pathogen, *Acremonium diospyri* (Crandall) W. Gams (Benade *et al.,* 1991;

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[2]Roux, C. Senior Mycologist, retired. Mycology section, PPRI, Pretoria, RSA.

[3]Brady, B.L. Senior scientist, retired.. Commonwealth Mycological Institute, Kew, Surrey, U.K.

[4]Sutton, B.C. Senior scientist, retired. Commonwealth Mycological Institute, Kew, Surrey, U.K.

[5]Gams, W., Professor Emeritus, CBS, Oosterstraat 1, Baarn, The Netherlands.

[6]Menge, J. Professor emeritus, Dept. of Plant Pathology, UC Riverside, California, USA.

Benade *et al*., 1992), and although morphologically similar, was found to be distinct in terms of cytoplasmic long chain carboxylic acids.

Descriptions of the symptomology according to Crandall (1939; 1945; Crandall and Baker, 1950), Wilson (1963), McRitchie (1979) and Holdeman (1998), of persimmon wilt, closely parallels that of GWD symptomatology, particularly in terms of the rapidity of death (two weeks from the onset of symptoms), canopy symptoms (rapid wilting and defoliation), spreading by root grafts, tissue discoloration (black streaks in the wood) and erumpent sporulation (pink spore masses of the fungus produced beneath the bark). The impact of persimmon wilt in the first half of the 20th century in the south eastern USA was clearly described by McRitchie (1979) in terms of the rapidity and virulence of the pathogen as quoted from his paper, "*The fungus, Cephalosporium diospyri, virtually eliminated persimmon from one of its main habitats, the central basin of Tennessee, and has been found south to Florida and west to Oklahoma. The pathogen is so efficient that it was once proposed as a biological control agent to eliminate unwanted native persimmons*". The description of the persimmon epidemic in the USA closely resembles the decimation of the guava industry in the Province of Mpumalanga as a result of GWD. Currently no acceptable level of commercial genetic tolerance to persimmon wilt has been identified in *D. virginiana* because the industry is highly reliant on the horizontally resistant Asian species (*Diospyros kaki* and *D. lotus)*.

Schroers *et al*.,(2005) undertook a thorough taxonomic review and reclassification of the GWD fungus, the palm wilt pathogen, *Gliocladium vermoesenii* and the persimmon wilt fungus, *Acremonium diospyri*, utilizing morphological characters and molecular technologies. The outcome was that he placed all these pathogens into the genus *Nalanthamala*. In this new

classification, the causal organism of GWD in South Africa and Taiwan was named *Nalanthamala psidii* (Biourge) Schroers to replace *Gliocladium vermoesenii*. A summary of those revisions are given in Table 1.1., and which have contributed to a clearer understanding of the taxonomy of the pathogens causing GWD globally.

Table 1.1. A summary of the taxonomic revisions to three closely related pathogens and their placement in the Genus *Nalanthamala* by Schroers *et al*., (2005)

Previous identity	Host	Disease	Geographic distribution	Proposed new name
Myxosporium psidii	Guava	Likubin (wilt)	Asia	*Nalanthamala psidii*
Gliocladium psidii	Guava	Guava Wilt	Southern Africa	*Nalanthamala psidii*
Gliocladium vermoesenii	Palm	Pink Rot	Americas, Asia, Australia, Europe	*Nalanthamala vermoesenii*
Acremonium diospyri	persimmon	Persimmon Wilt	North America	*Nalanthamala diospyri*

GWD spread quickly in South African and Taiwanese commercial varieties due to inadequate host resistance (Grech *et al*., 1987). The South African industry was exclusively based on one commercial cultivar; Fan Retief, a multi-purpose cultivar that is highly susceptible to the disease (Grech *et al*., 1985, Du Preez and Welgemoed 1988).

Over the course of this study, guava wilt disease was not detected in any sites visited in the Americas and is currently limited to South East Asia and Southern Africa (Fig. 1). Outside of any country (or region) that had previously reported GWD, the disease was only detected in

Malaysia (where at the time writing this study, it has not been officially reported to be present). In Taiwan the disease spread from its first detection point in 1926 to all production regions on the island over a period of 50 years. A report of GWD occurring in Xiamen, Fujian, China in 2002 was investigated by the author and found not to be valid.

Fortunately, at present the Cape production areas of South Africa are free of GWD and the movement of plant material to those areas from the northern and eastern guava growing regions has been prohibited since 1985 (Grech and Rijkenberg, 1987). The situation in Taiwan followed a similar pattern except that four commercial varieties were generally used, all of which were highly susceptible to the GWD pathogen. In Taiwan the ubiquitous distribution of GWD resulted in the average orchard replacement cycle being reduced to eight to ten years as compared to 25-30 years in South Africa when the disease was first identified in the early eighties (Grech, 1987). Since that time, average tree age has diminished in South Africa as GWD has spread. The reduced orchard longevity resulting from GWD infection, coupled to increased costs of orchard replacement have significantly increased production costs. In most cases, where orchards have been afflicted with GWD, guavas are not re-established on the lands due to the threat of rapid re-infection of the new orchard.

1.5   Thesis Research

The studies reported herein were field orientated and focused on multiple research objectives with particular emphasis (but not limited to): the global distribution of GWD and *N. psidii* strain collections; confirmation of the taxonomic status of the causal organism; histopathology of GWD; characterization of the disease symptomology and the development of a GWD rating

scale; understanding the etiology of GWD, and the disease cycle; determination of the host range of *N. psidii* within the Myrtaceae, germplasm collections and screening for *N. psidii* resistance and the development of management strategies for GWD. These studies were conducted over several years in multiple international locations within both commercial orchards and dedicated research sites. A significant amount of work is not reported in this thesis as it falls outside of the scope of this work or is to voluminous to be included. This thesis consists of thirteen distinct chapters addressing the aforementioned objectives.

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CHAPTER 2: ISOLATION OF THE CAUSAL ORGANISM OF GUAVA WILT DISEASE, AND FULFILLMENT OF KOCH'S POSTULATES

ABSTRACT

Extensive collections of guava root and stem tissues were undertaken from trees expressing guava wilt disease (GWD) symptoms in various production areas in Southern Africa and Taiwan. Fungal isolation procedures from diseased guava xylem tissues resulted in the consistent recovery of a fungus that had identical cultural morphology from all geographical regions that were sampled. All isolates were pathogenic to the major guava cultivar grown in South Africa (cv. Fan Retief), when inoculated in the main stems of greenhouse plants grown under diurnal lighting conditions at 25°C or 33°C, and when utilizing mycelial or conidial (or both) inocula. The fungus was found to require wounding for infection. It produced two types of conidia in culture (Type 1 and Type 2). The fungus was successfully re-isolated from inoculated tissues and Koch's postulate was fulfilled. In active woody tissues, the fungus moved acropetally more rapidly than basipetally. The fungus morphologically was identical to that described from Taiwan, *Nalanthamala psidii* (Biourge) Schroers, which causes a wilt of guava in that country.

2.1 INTRODUCTION

GWD is the most serious disease of guava globally (Hong *et al*., 2015) and caused by *Nalanthamala psidii* (Biourge) Schroers. In the course of this study, the disease was found to be present in the production regions of Mpumalanga, Limpopo (South Africa), Swaziland and Southern Mozambique (Leu and Kao, 1979; Grech, 1985, 1990). In addition, infected guava tissue was also collected from several locations in Taiwan, Republic of China. The aim of this study was to isolate the causal organism, fulfil Koch's postulates and investigate the role of wounding in terms of infection by the GWD pathogen.

2.2   MATERIALS AND METHODS

2.2.1   Collection procedures

Diseased guava trees with symptomatology resembling those described by Grech (1983, 1985), were identified. Tissue samples (wood sections, 30cm from above the soil line, 1cm thick and cut perpendicularly across the trunk), were collected from guava trees exhibiting typical early symptoms (Leu and Kao, 1979) of GWD from South Africa, Taiwan, Swaziland and Mozambique (Table 2.1). The samples were refrigerated at 10^0C for not more than two days prior to processing. The trunk sections were cut longitudinally along the grain of the wood, across the entire diameter of the section, into approximately $1cm^3$ cubes, prior to isolation procedures. Additional trunk sections were collected from healthy and infected trees by cutting a section 20 cm in length from the main trunk or scaffold branch. Some of these sections from diseased trees exhibited typical bark blistering (Fig. 2.2), which contained sporodochial structures (Leu and Kao, 1979; Grech 1985).

2.2.2   Isolation procedures

The trunk wood sections from healthy diseased and recently dead trees (three days *post-mortem*) were surface sterilized by placing them in 0.5% sodium hypochlorite (NaOCl) for 2 minutes, followed by rinsing in sterile deionized water (SDW). These sections were then aseptically pared into $0.5mm^3$ pieces and placed in 9 cm diameter Petri dishes of half strength potato dextrose agar [(PDA) Difco Inc., Sparks, Nevada, USA], amended with 100 mg.L^{-1}

penicillin (Merck Chemicals, Halfway House, Johannesburg, RSA). Trunk sections from diseased trees that did not have evidence of bark blisters or sporodochial development were placed in a humid chamber at 95% relative humidity for 3 days at 28°C°C. From conidial masses that formed on the cut ends, conidia were removed using a sterile wire needle, and plated onto half strength corn meal agar (Difco Inc., Sparks, Nevada, USA) amended with 100 mg.L^{-1} penicillin (CMA+P). Trunk sections with evidence of unruptured bark blisters were examined and the outer bark peeled away to expose the sporodochial structures. Conidia from the sporodochia were removed on a sterilized metal needle and plated onto half strength CMA+P.

Tissue isolates in Petri dishes were incubated at 25°C°C under a 12h day/12 night lighting regime for five days. Color 95, TLD 18 W cool fluorescent daylight tubes (Philips Pty, Ltd, Johannesburg) were used to illuminate the cultures (placed 30cm below the lamps), emitting a nominal visible spectral intensity of approximately 2.16 x 10^{-3} W cm^{-2}. The ballasts for the lamps were mounted outside the incubators to avoid local heating effects on the cultures.

Petri dishes were observed after three days for evidence of microbial growth. If detected, the microbial culture was sub-cultured onto half strength CMA and incubated as above. After 5 days in culture, the predominant isolate(s) were selected, and re-plated onto half strength CMA. Hyphal tips were isolated from these cultures using a fine point needle and plated onto half strength CMA. After seven days of incubation under the same conditions as described above, the fungal cultures were put into storage as slant cultures (CMI, 1983) under oil, as well as storing some isolates in a lyophilized state. Cultures were assigned a code (Table 2.1) and

representative isolates sent to mycological identification centers, including The Commonwealth Agricultural Bureau International Bioscience, Egham, U.K. and the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

2.2.3　Fulfilment of Koch's Postulates

Several hundred guava plants (cv. Fan Retief) were propagated by modified air layering (also known as marcottage), as described by Hartmann *et al,.* (1983). The modified technique comprised of removing a 25 mm section of bark from a branch approximately 12 mm in diameter and spraying it with100 mg.L^{-1} indole butyric acid (Merck, Halfway House, South Africa), dissolved in an ethanol:water:glycerol (50:25:25 ratio) solvent, over the cut surface immediately before planting it into the growing medium. Although labor intensive and slow, the technique is suited to humid regions. Further, guava is difficult to propagate through leaf cuttings (Fitchett, 1986, pers. Comm[2]; Hartmann *et al.,* 1983; Vos *et al.,* 1998).

Approximately 150 fungal isolates from wilted guava trees at various geographic locations (Table 2.1), and putatively assumed to be infected with GWD, were each utilized in the fulfillment of Koch's postulates. Of these, two representative GWD type culture isolates, one from Taiwan and the other from South Africa (designated CSF t226 and CSF 221), were utilized for subsequent further studies. All isolates were sub-cultured onto PDA in Petri dishes and incubated under conditions as described earlier. After 8 days incubation, a 5mm diameter

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[2] Fitchett, M., Previously of ITSC, Nelspruit, RSA

plug of mycelium from 5 mm behind the advancing front of the expanding colony on PDA was

removed. These plugs were inserted into a 2cm^2 bark flap on the trunks, 10 cm above the soil

line, of 1 year old clonal guavas (cv. Fan Retief). The wound was gently wrapped with

Parafilm$^®$ (Pechiney Packaging Inc., New Jersey, USA, formally American National Can,

Chicago, USA), followed by sealing with masking tape (3M, Minnesota, USA), as shown in

Fig. 2.1.
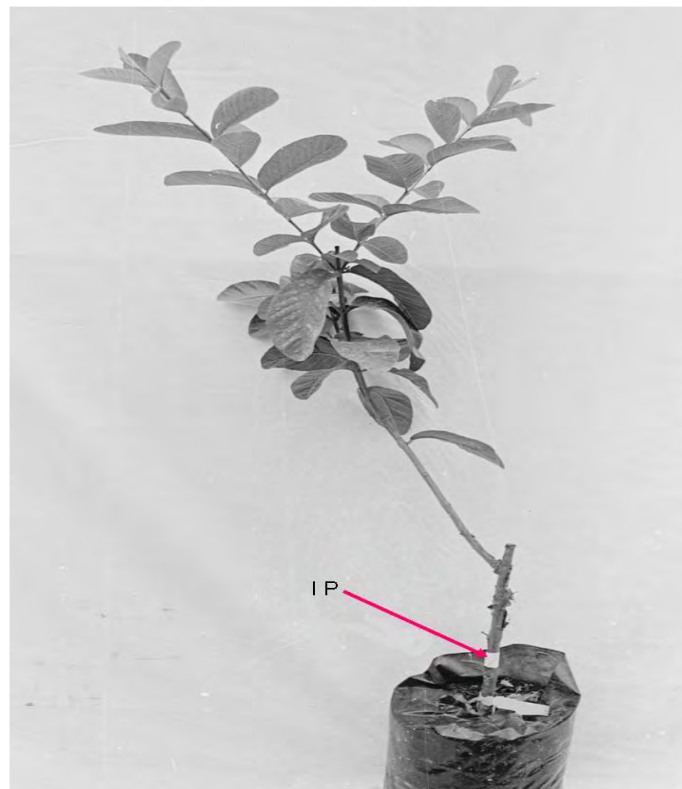
Figure 2.1. Artificial inoculation of a 1 year old guava plant (cv. Fan Retief) showing the
inoculation point (IP).

Identical fungal plugs were similarly placed against the unwounded trunk of Fan Retief guava trees and also gently wrapped with Parafilm®. In both inoculation groups, two additional Control treatments were applied: sterile PDA plugs were placed on the unwounded trunk, or in a bark flap on the guava plants.

2.2.4   Preliminary pathogenicity tests of different conidial types

It was observed that the cultural phenotypic characteristics of *N. psidii* isolates that were grown on rich culture media such as PDA and repeatedly sub-cultured, underwent a significant change in macroscopic cultural morphology (usually within 5-6 sub-cultures). Hence, in addition to the pathogenicity testing mentioned above, an isolate of a sub-culture of a South African GWD isolate (CSF 207) that exhibited gross morphological differences from the type cultures was compared to Isolate CSF 221, in terms of pathogenicity. CSF 207 was used to inoculate ~~five~~ ten clonal guava plants, as described earlier, to determine if these morphological changes occurring in artificial culture were indicative of a change of pathogenicity.

The inoculated plants were placed in a greenhouse chamber at a constant 33°C. All plants were under natural lighting and randomized into single tree plots. Each treatment was replicated five times. Plants were monitored over the course of four to five months for disease symptoms (growth abnormalities, stem necrosis, wilting and eventual death). During the course of the experiments, plants that died were removed and six wood sections (three above the inoculation point and three below, at 3 cm intervals, starting at the point of inoculation) were removed from the trunk for pathogen fungal re-isolations (no later than 24-48 hours after plant death).

At the end of the 4 month assessment period all wounded plants (and unwounded guava trees inoculated with the *N. psidii* isolates) were sectioned similarly for fungal isolation and examined under the light microscope for the presence of fungal colonization.

The two GWD type isolates (CSF t226 and CSF 221) both produce two conidial types (Chapter 3). The two isolates and their two conidial types were tested for their comparative pathogenicity at two incubation temperatures. Isolates were single spored by aseptically transferring 20 individual conidia with a sterilized fine pointed metal needle to two Petri dishes of acidified water agar [WAA, 20g agar (Difco Inc., Sparks, Nevada, USA) in 1L of deionized water, adjusted to a pH 5.0] and incubated as above. Isolates derived from single spores were grown under incubation conditions, as described in Chapter 4, which favored Type 1 or Type 2 conidial production. The Type 1, hydrophobic conidia are primarily produced on aerial conidiophores (Chapter 3, Figs. 3.8, 3.9 and 3.10), and were isolated by inverting the profusely sporulating Petri plate culture and contacting the basal plate with a vibratory spatula for 15 seconds. This procedure was applied to 20 Petri plate cultures. The lids were carefully removed under sterile conditions. The conidia were harvested by sequentially washing each plate with the same 10 ml of SDW plus a sterilized non-ionic wetting agent (0.1% Tween[20], (Merck Chemicals, Halfway House, Johannesburg, RSA). In addition, each Petri plate lid was lightly rubbed with a sterilized camel-hair brush to loosen adhered conidia. The conidial suspension was checked for spore density and purity of Type 1 conidia by direct microscopic examination.

Type 2 conidia are hydrophilic and are predominantly produced on prostrate conidiophores in gelatinous heads (Chapter 3, Figs. 3.8, 3.10 and, 3.12). Petri plate cultures of the *N. psidii*

isolates were established on 20 plates of half strength PDA. Sterilized glass plates were placed over the surface of the actively growing cultures and the Petri lids placed on the Petri dishes. These plates were incubated in darkness for 2 weeks at 20°C. The Type 2 conidia were harvested under aseptic conditions by rinsing the 20 Petri dish cultures sequentially with the same 10 ml of SDW containing a sterilized non-ionic wetting agent (0.1% Tween[20]). The glass plates and the surface of the cultures were lightly rubbed with a sterilized camel-hair brush to loosen adhered conidia. The suspension was then examined microscopically to determine the density and purity of the Type 2 conidial suspension. Purification of the Type 2 conidial suspension was further undertaken by filtering the conidial suspension under partial vacuum through a Millipore filter with a pore aperture slightly larger than the longest axis of the smaller Type 1 conidium (5 μm). The larger Type 2 conidia were then recovered by inverting the Millipore filter and back flushing with an additional 100 ml of SDW plus wetter to release the adhered larger conidia. The viability of conidia subjected to these treatments were assessed by single sporing a sub-sample of them onto water agar (amended with 100 mg.L^{-1} penicillin) and observing whether or not germination occurred. Following spore separation, conidial densities were adjusted to approximately 1×10^5 conidia.ml^{-1}, using a haemocytometer.

Guava plants (cv. Fan Retief) were inoculated as previously outlined except that the inoculum in this case was 1 ml of the respective Type 1 or Type 2 conidial suspension placed inside a 2 cm bark flap on the trunk of the test plants. Control plants were similarly treated except that the respective spores were applied to unwounded tissues. Treatments were replicated 5 times, each replicate comprising a single plant. All treatments were placed in temperature controlled greenhouses under ambient light. One group of treatments was placed in a greenhouse chamber

held continuously at 25^0C while the other group of treatments was placed in a similar greenhouse chamber held at 33^0C. Plants were monitored over four months for GWD symptoms, as prior unpublished work by Grech had shown that tree mortality was slower when conidia were used as the inoculum, compared to mycelial agar plugs. An additional group of treatments were incubated in the greenhouse in the same manner as for the conidial inoculations except that mixed Type 1 and Type 2 conidial suspensions were compared to mycelial inoculations, as described earlier, to test for relative rapidity of induction of plant mortality by mixed Type and Type 2 conidial inoculum versus pure Type 1 or Type 2 conidial inoculum.

2.3   DATA ANALYSES

Data were analyzed by one or two way analysis of variance and where appropriate, treatment means were separated using the Waller-Duncan *k*-ratio *t* test (Little and Hills 1990).

2.4   RESULTS

2.4.1 Isolation of the causal organism

Incubation of trunk sections from diseased field-collected guava trees resulted in profuse fungal growth from xylem tissues immediately adjacent to cambium tissues (Fig. 2.2). Shortly after tree death, bark blisters appeared on the trunk and branches, which then ruptured and released conidia from sporodochial structures (Fig. 2.2). Initially, mycelial growth was emanating exclusively from the active xylem (outer wood) on cut trunk sections. Tissue isolations from GWD infected guavas from all geographic locations resulted in a high recovery (> 85%) of identical fungal isolates.

Fungal growth was not initially observed growing from the heart wood (inactive xylem) until after several days of incubation of the trunk sections of recently dead guava trees (Fig 2.2). Active (living) xylem tissue isolations from the trunks of diseased guavas, in all geographic locations tested, consistently gave rise to fungal isolates that were morphologically identical and shared similarities with species in the genus *Nalanthamala.*
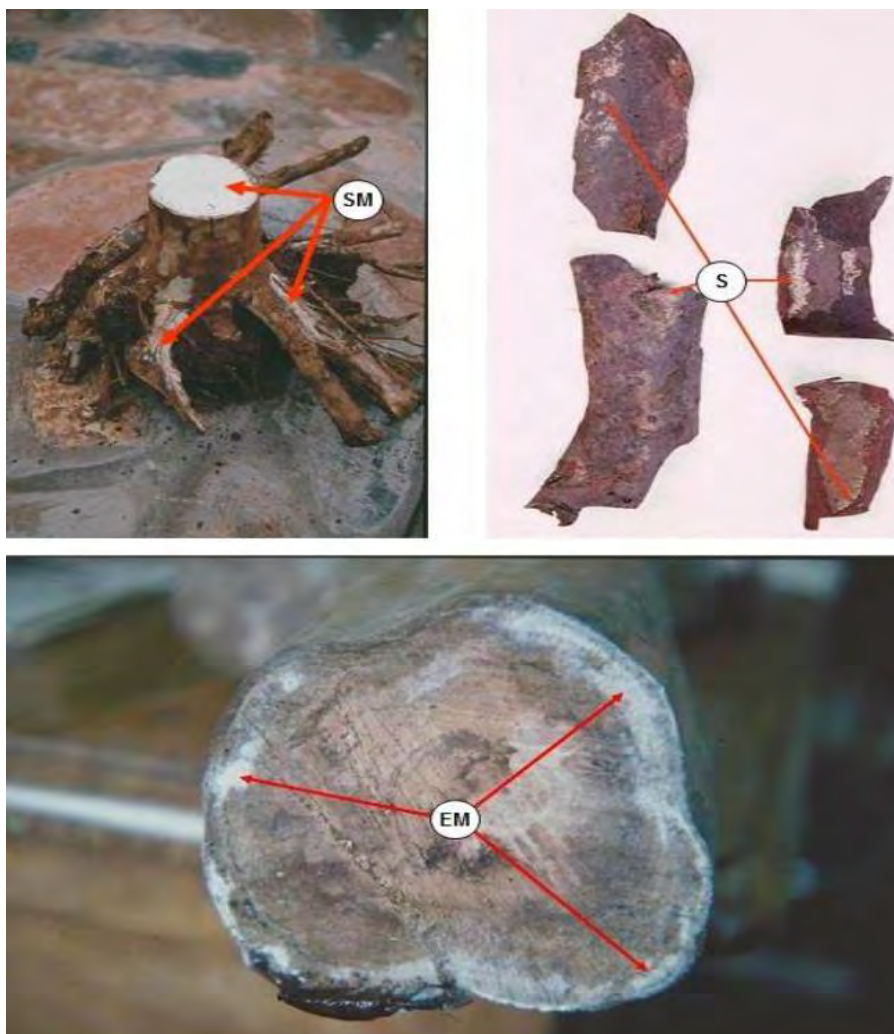
Figure 2.2. Sporulation on various tissues from a recently dead guava tree (3 days *post mortem*).

SM   = Sporulating mycelia emanating from cut or abraded surfaces.
S   = Sporulating mycelia emerging from sporodochia beneath the bark.
EM   = Emergent mycelia from active xylem vessels in the outer trunk regions.

2.4.2 Pathogenicity tests and fulfillment of Koch's Postulate

Isolates from GWD-infected guava trees, from all geographic locations induced plant mortality when inoculated into wounded test plants. A fungus was consistently recovered from infected tissues that was identical to the fungus used to inoculate the plant. Plant mortality was only observed where the *N. psidii* isolates (conidia or mycelium) were wound inoculated into the guava tree clones (Tables 2.1 and 2.2).

Table 2.1 Guava wilt distribution in selected regions, predominant fungal genera isolated and pathogenicity

Location	Dates	CSF accession no.[a]	Putative fungal identification[b]	Pathogenic on guava[c]
South Africa				
Malelane	1982-87	CSF 112-131	*Nalanthamala*	Yes
Louw's Creek,	1982-88	CSF 202-225	*Nalanthamala*	Yes
Kaapmaiden	1983-87	CSF 307-312	*Nalanthamala*	Yes
Barberton	1984-88	CSF 356-368	*Nalanthamala*	Yes
Nelspruit	1985-90	CSF 402-434	*Nalanthamala*	Yes
Brondal	1985-90	CSF 507-526	*Nalanthamala*	Yes
Hoedspruit	1986-91	CSF 607-612	*Nalanthamala*	
Levubu	1987-92	CSF 703-710	*Nalanthamala*	Yes
Swaziland				
Manzini	1988-95	CSF s7-10	*Nalanthamala*	Yes
Mozambique				
Maputo	1989-91	CSF m3	*Nalanthamala*	Yes
Taiwan				
Taichung.	1987	CSF t201-203	*Nalanthamala*	Yes
Ta Sir	1987	CSF t204-209	*Nalanthamala*	Yes
Tainan	1987	CSF t210-218	*Nalanthamala*	Yes

Fengshung   1987, 2000   CSF t218-229   *Nalanthamala*   Yes

[a] Fungal isolate collection codes, ITSC, Nelspruit. [b]Based on cultural morphological characteristics of predominant fungi isolated from diseased guavas and determined to be pathogenic. [c]Ten single plant (cv. Fan Retief) replicates were inoculated with a type culture fungal isolate as described above. Treatments were assessed over a four month period for any symptoms of GWD and ultimately plant mortality.

Fungal colonization of the xylem vessels was observed to occur significantly faster acropetally than basipetally (Fig. 2.3). In most of the inoculated trees, at the time of tree death, the *N. psidii* isolates were recovered in lower frequencies from the xylem tissues below the trunk inoculation point as compared to recoveries from above the inoculation point. None of the Control trees developed GWD.
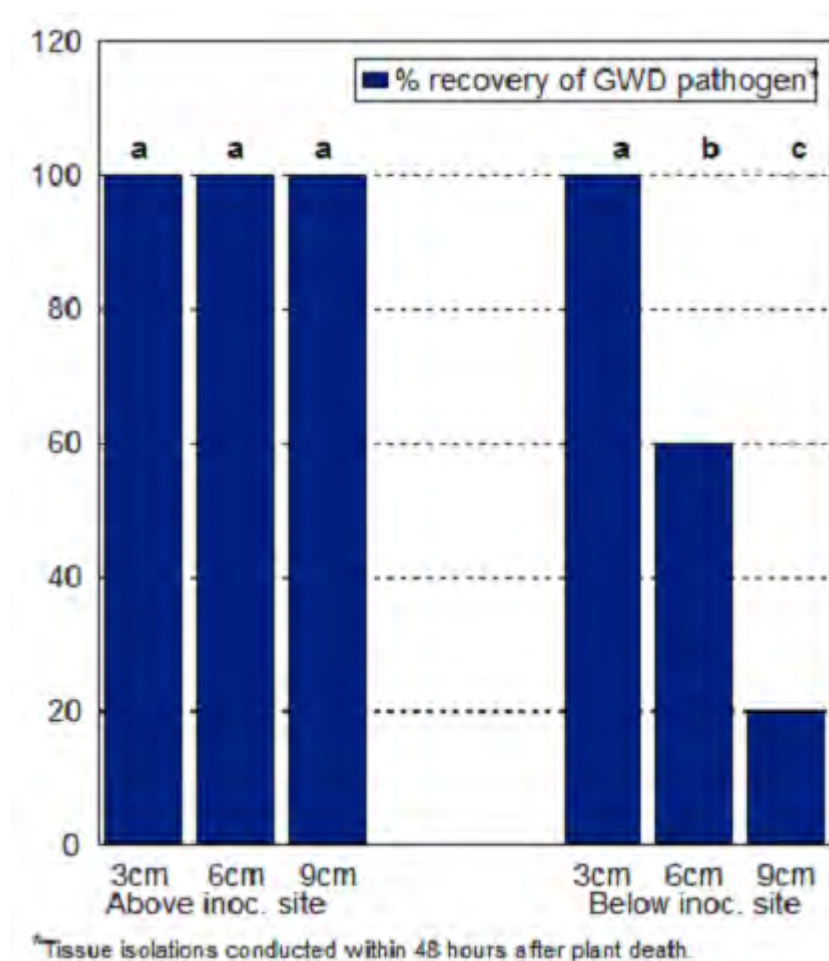
Figure 2.3. The influence of distance from the trunk inoculation point on the recovery of the GWD fungus. Tissue sections were excised from the diseased trees no later than 24 hr after plant death.

Unwounded trees inoculated with the *N. psidii* isolates (conidia or mycelium) remained healthy over the course of the experiment (Table 2.2).

2.4.3 Preliminary pathogenicity tests of different conidial types

Both conidial types of both isolates were found to be infective at 25°C°C and 33°C°C when wound inoculated into guava tissues (Table 2.2). Inoculations that were attempted without wounding were unsuccessful in inducing plant death.

Table 2.2. Pathogenicity on guava of the Type 1 (T-1) and of the Type 2 (T-2) conidia produced by the *Nalanthamala psidii* isolates, CSF 221 (South African) and CSF t226 (Taiwanese), when inoculated on to wounded or unwounded tissues

Isolate	Number of dead trees (of 5)[a]			
	T-1		**T-2**	
	25°C	**33°C**	**25°C**	**33°C**
		Wounded		
CSF 221 (RSA)	5 (2) a	5 (4) a	5 (4) a	5 (3) a
CSF t226 (ROC, Taiwan)	5 (2) a	5 (5) a	5 (3) a	5 (3) a
		Unwounded		
CSF 221 (RSA)	0 b	0 b	0 b	0 b
CSF t226 (ROC, Taiwan)	0 b	0 b	0 b	0 b

[a] Five replicates per treatment, trees assessed at 2 mo. and 4 mo. after inoculation (figures in parenthesis). Figures outside of the parentheses in the same column followed by the same letter are not significantly different from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.01). RSA = Republic of South Africa, ROC = Republic of China.

Isolate CSF 207 induced plant death in guava, albeit at a slower rate than Isolate CSF 221.

When re-isolated from guava, the cultural morphology of this isolate reverted to that of the

type culture, CSF 221, and induced plant death at similar rates to Isolate CSF 221 in subsequent inoculations of guava trees.

2.5   DISCUSSION

All *N. psidii* isolates were pathogenic to guava (cv. Fan Retief), in these experiments (Table 2.1). Inoculated guava plants expressed leaf discoloration, leaf drop and wilting symptoms within approximately 5-6 weeks. All plant died within four months of inoculation, although many plants died sooner. *Nalanthamala psidii* isolates did not induce plant mortality unless the plants were wounded prior to inoculation, suggesting that this fungus is a wound pathogen (Table 2.2 and Chapter 4, Fig 4.5), a property shared with the persimmon wilt fungus (Crandall, 1939; Crandall and Baker, 1950). Persimmon wilt is caused by the fungus *Nalanthamala diospyri* (Crand.) Schroers and M.J. Wingf., 2005. Previously it was identified as *Acremonium diospyri* (Crandall) W. Gams (and prior to that, identified as *Cephalosporium diospyri* Crand.). The Genus *Acremonium* is reported to contain species that produce two conidial types (Giraldo, *et al.*, 2014), a property shared with the GWD pathogen.

Subsequent re-isolation of the fungus from inoculated guava plants fulfilled Koch's Postulates. These results are in agreement with those of Leu and Kao (1979), Schroers *et al.*, (2005) and Athipunyakom and Luangsaard (2008).

Both types of conidia produced by the GWD pathogen were infective only when applied to a wound. They appeared to have different temperature optima, which is important for the epidemiology of the pathogen, allowing for infection over a broader temperature range. In trunk inoculations, the GWD pathogen moved faster acropetally than basipetally (Fig. 2.3), a common phenomenon in vascular wilting fungi (Bruehl, 1987).

The result that Isolate CSF 207 induced plant death in guava at a slower rate than Isolate CSF 221, reflecting reduced pathogenicity. However, when passed through and re-isolated from guava, the cultural morphology of Isolate CSF 207 reverted to that of the type culture, CSF 221, and at a rate of pathogenicity similar to Isolate CSF 221, which suggests that the loss of pathogenicity was epigenetic rather than a permanent mutation.

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CHAPTER 3: IDENTIFICATION, TYPE CULTURE COMPARISONS, MICROSCOPY AND GENOMIC ANALYSIS OF THE PATHOGEN, NALANTHAMALA PSIDII

ABSTRACT

The taxonomy of the causal fungus of guava wilt disease (GWD) has over the past several decades been challenging. The GWD fungal pathogen isolates from Asia and South Africa are morphologically identical. This pathogen has been classified as *Nalanthamala psidii* (Biourge) Schroers. Microscopic studies identified unique features of this fungus such as two distinct conidial types (Type 1 and Type 2), which are produced on different host tissues and which have different germination temperature optima. Type 1 and Type 2 conidia were successfully separated and were both found to be highly infective, inducing plant death usually within 4 months after wound inoculation. Other important etiological attributes of *N. psidii* were observed and examined, such as: prolific conidiogenesis on moribund and/or dead host tissues of aerial and water disseminated propagules; two distinct types of hyphae; competent host xylem colonization; chlamydospore production in axenic culture; and with the production of endohyphae in culture and within the host's xylem tissues, which is an important adaptation for vascular wilt inducing fungi. Scanning electron microscopy revealed that *N. psidii* breached xylem vessel pits utilizing morphologically distinct slender hyphae. Extensive tyloses were observed in above ground infected active xylem (sapwood) tissues but not in root tissues, where amorphous agglomerations were more common. Pathogenically, *N. psidii* shares significant similarities with the persimmon wilt fungus and the palm pink rot fungus, both of which are now classified in the genus *Nalanthamala* and which have been described as *Nalanthamala diospyri* (Crandall) Schroers and *Nalanthamala vermoesenii* (Biourge) Schroers, respectively. Genomic analyses utilizing the D2 region of the 28S large subunit genome indicated that these three plant pathogens were closely related and shared significant sequence homology. These studies supported the placement of the guava wilt pathogen in the genus *Nalanthamala*.

3.1   INTRODUCTION

Guava wilt disease (GWD) was first reported in South Africa and Swaziland by the author in 1982 (Grech, 1983; Grech 1985a, 1985b; Grech and Rijkenberg, 1987) and was initially described as being caused by *Septofusidium elegantulum* (Pidopl.) W. Gams. *Septofusidium* is an obscure genus of three species, all similar to *Acremonium* (Gams, 1971). Indeed, *Acremonium diospyri* (Crandall) W. Gams (previously identified as *Cephalosporium diospyri*) has been described as causing a wilt of persimmon (*Diospyros virginiana*) and sycamore *(Acer pseudoplatanus*), diseases that share many similar symptoms to GWD (Crandall, 1945; Crandall, and Baker,. 1950; Wilson, 1963; MacRitchie 1979; Leininger *et al.*, 1999). In Taiwan the wilt disease of guava (abbreviated in this study as TGWD), as reported by Kurosawa (1926), was described as being caused by *Myxosporium psidii* Sawada and Kurosawa (Hsieh *et al.*, 1976), a genus previously determined to be no longer valid (Hohnel, 1915). Brady (1983, personal communication) identified a South African and a Taiwanese isolate of the GWD pathogen as *Gliocladium vermoesenii* and *G. catenulatum* (IMI 279785 and 277770), respectively. Gams (1985, personal communication), on the other hand, believed this to be a new fungus resembling *Nalanthamala madreeya*, a fungus not known in culture. Menge (1994, personal communication) believed that the GWD fungus should be placed in the genus *Gliocladium*. Preliminary studies (Grech, 1987 and 1988) indicated that the Taiwanese and South African GWDs were similar and were both caused by a species of *Gliocladium*. *Gliocladium vermoesenii* (Bourge) Thom has been reported as causing severe wilt diseases on a number of palm species in several countries (Feather, *et al.*, 1979; Feather, 1982).

The review by Schroers (2005) of the GWD fungus, the palm wilt pathogen, *Gliocladium vermoesenii* and the persimmon wilt fungus, *Acremonium diospyri*, utilized morphological characters and molecular technologies. This study significantly clarified the taxonomic status, as well as providing a clearer understanding of these three pathogens. A chronology of the nomenclature assigned to the GWD pathogen is given in Table 3.1, including comments as to the limitations of prior putative identifications ascribed to this fungus.

Table 3.1. A Chronology of the Nomenclature Assigned to the Guava Wilting Pathogen

Location	Date	Collector, isolated by, depositor[a]	Putative I.D (and identifier)	Identification anomalies
Taiwan	1926	E. Kurosawa	*Myxosporium psidii* (Kurosawa)	Genus not valid
Taiwan	1979	L.S. Leu	*Myxosporium psidii* (Leu)	Genus not valid
South Africa	1981	B.Q. Manicom	*Paecilomyces* (v.d. Westhuizen)	Monoconidial
South Africa	1982	N.M. Grech	*Septofusidium sp.* (C. Roux)	Monoconidial
South Africa	1983	N.M. Grech	*S. elegantulum* (C. Roux)	Monoconidial
South Africa	1983	N.M. Grech	*Gliocladium* (Grech)	Monoconidial
South Africa	1983	N.M. Grech	*Gliocladium vermoesenii* (Sutton)	Type culture dissimilarity
Taiwan	1983	N.M. Grech	*Gliocladium catenulatum* (Brady)	Monoconidial
South Africa	1983	N.M. Grech	*Gliocladium vermoesenii* (Brady)	Type culture dissimilarity
Philippines	1984	Quimio	*Acremonium* sp. (Quimio)	Type culture dissimilarity
South Africa	1985	N.M. Grech	*Nalanthamala madreeya* (Gams)	Not known in culture
South Africa	1990	N.M. Grech	*Gliocladium sp.* (Menge)	Monoconidial
South Africa	97-2002	M.H. Schoeman	G*liocladium vermoesenii* (Schoeman)	Type culture dissimilarity
South Africa	2005	H.J. Schroers	*N. psidii* (Schroers)	rDNA confirmed I.D.
South Africa	2007	N.M. Grech	*N. psidii* (Grech)	rDNA confirmed I.D.
India	2007	A.K. Misra	*Gliocladium roseum* (Misra)	Monoconidial

Roux, C., Senior Mycologist and G.C.A van der Westhuizen, both retired. Mycology Section, PPRI, Pretoria, RSA; Brady, B.L. Senior Scientist, retired. Commonwealth Mycological Institute, Kew, Surrey, U.K.; Sutton, B.C. Senior Scientist, retired. Commonwealth Mycological Institute, Kew, Surrey, U.K.; Gams, W., Professor Emeritus, CBS, Oosterstraat 1, Baarn, The Netherlands.; B.Q. Manicom, Deputy Head, retired, ITSC, Nelspruit; E. Karosawa, TARI, Taichung, Taiwan, deceased. L.S. Leu, Senior Scientist, retired, Fengshan Tropical Research Station, Taiwan; M. H. Schoeman, Senior Researcher ITSC, Nelspruit, South Africa; H.J. Schroers, CBS, Utrecht, The Netherlands; Menge, J. Professor Emeritus, Dept. of Plant Pathology, UC Riverside, California, USA.

The purpose of this study was to further investigate the identity of the causal organisms of GWD in South Africa (GWD) as well as from Taiwan (TGWD) and to re-confirm the taxonomic status of these organisms. The two type cultures of the TGWD and GWD isolates of *N. psidii*, one from Taiwan and the other from South Africa, were designated CSF t226 and CSF 221.

3.2   MATERIALS AND METHODS

3.2.1   Fungal type cultures

Representative isolates of the GWD pathogen from South Africa and Taiwan were compared to type cultures of fungi that were believed to be related to the GWD pathogen and/or fungi that the GWD pathogen had been previously taxonomically assigned. These type cultures, accessed from various persons and mycological institutes, as given in Table 3.2., were used for morphological and pathological comparisons.

Table 3.2. Fungal Type Cultures Used for Taxonomic Comparisons with the *Nalanthamala psidii* Isolates and their Pathogenicity to Guava

Isolate	Source	Host	Pathogenic on Guava*
Gliocladium album	IMI 40084	*Physarum*	No
G. virens	IMI 177777	*Ricinus communis*	No
G. viride	IMI 96735	*Populus spp.*	No
G. deliquescens	IMI 69036	*Eucalyptus regnans*	No
G. flavofuscum	IMI 100714	Soil	No
G. roseum	IMI 177664	*Triticum*	No
G. catenulatum	IMI 57402	*Pyrus*	No
G. vermoesenii	IMI 40231	*Citrus*	No
Paecilomyces sp.	Marasas, M.R.I.	Unknown	No
Acremonium diospyri	ATCC 9066	*Diospyros virginiana*	No
G. vermoesenii[a]	IMI279785 (CSF221)	*P. guajava*	Yes
G. vermoesenii[a]	CSF 207	*P. guajava*	Yes/low virulence
G. vermoesenii[a]	IMI 277770 (CSF t226)	*P. guajava*	Yes

IMI= CABI Bioscience (formally International Mycological Institute), Egham, UK., **ATCC** = American Type Culture Collection, Manassas, Virginia, U.S.A., **PREM**=ARC, National Collection of Fungi (formally Mycological Research Unit), Pretoria, R.S.A. **MRI** = Medical Research Institute, Tygerberg, R.S.A. **CSF** = author's collection of *N. psidii* isolates held at the CSFRI (now ITSC). * Five cloned guava plant replicates (cv. Fan Retief) were inoculated with a type culture fungal isolate, as previously described. Plants were assessed at 25^0C and 33^0C over a four month period for any symptoms of wilting or vascular necrosis. [a] Putative identification of the GWD pathogen (personal communication, Brady, 1983). CSF 207$_{NP}$ is a previously pathogenic GWD isolate that in culture became predominantly non-pathogenic.

Septofusidium elegantulum (Pidopl.) W. Gams has been described as being hard to culture (Gams, 1971) and during these experiments, cultures were not available. Similarly, the type species of the genus *Nalanthamala*, *N. madreeya,* was not known in culture when these experiments were being conducted.

Fungal type cultures were also initially plated onto half strength cornmeal agar [CMA (Difco, Sparks, Nevada, USA)] and incubated at 25°C under a 12h day/12 night lighting regime for five days. Color 95, TLD 18 W cool fluorescent daylight tubes (Philips Pty, Ltd, Johannesburg) were used to illuminate the cultures (placed 30cm below the lamps), emitting a nominal visible spectral intensity of approximately 2.16×10^{-3} W cm^{-2}. The ballasts for the lamps were mounted outside the incubators to avoid local heating effects on the cultures.

Petri dishes were observed after three days for evidence of microbial growth. If detected the microbial culture was sub-cultured onto half strength CMA and incubated as above. After five days all cultures were inspected for production of conidia, and if conidia were found, then the isolates were single spored by aseptically transferring 20 individual conidia with a sterilized fine pointed metal needle into two Petri dishes of acidified water agar [WAA, 20g agar (Difco, Sparks, Nevada, USA) in 1L of deionized water, adjusted to a pH 5.0] and as above. If conidia were absent, then hyphal tips were taken instead. These cultures derived from single conidia (or hypha tip cloned) were compared for homogeneity in their cultural morphology. Deviants were discarded where they differed from what was expected in the type cultures, according to taxonomic keys (Raper and Thom, 1949; Gams, 1971; Pitt, 1979; Domsch *et al.*, 1980; Morquer *et al.*, 1981; von Arx, 1981; Peberdy, 1987) or where replicate isolates exhibited

substantial cultural or morphological differences (as for the *N. psidii* isolates) from the majority of the cloned isolates.

3.2.2 Microscopy

Hyphal tips from single spore isolates (or from hyphal tips in the absence of conidia) were transferred to PDA and incubated as above. After an eight day incubation period, the fungal cultures were compared morphologically and pathogenically (on guava) with one another (Tables 3.2, 3.3 and 3.4), with particular emphasis on conidial types, conidial dimensions, hyphal types, and conidiophore morphology. Light microscope and scanning electron microscope studies were also undertaken on the two *N. psidii* isolates. Specimens for scanning electron microscopy were initially prepared using a modification of the technique of Gaudet and Kokko (1984). Sections of approximately 50mm^3 in volume were excised from an actively growing five day old, half strength CMA culture. The agar sections were placed upright on the inside of a 9cm glass Petri dish lid prior to vapor fixation. The lid was then placed over the bottom portion of the glass Petri dish which contained 10 ml of a 10% solution of glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.2) for 12 hours at room temperature. These sections were then further fixed for 4 hours by immersion in 4% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.2) at 6°C and placed under a partial vacuum. These sections were buffer rinsed and post-fixed in 2% osmium tetroxide in cacodylate buffer for an additional 24 hours (or until uniformly blackened) at 6°C under vacuum. The sections were then buffer rinsed three times, dehydrated in an acetone series [10%, 30%, 50%, 70%, 90%, 95% acetone in water (w/v) and 100%] with a residence time in each acetone dilution of 20 minutes. The

specimens were further sectioned. The sections were then placed in a critical point dryer and flushed with carbon dioxide until no odor of acetone was detected. The specimens were then critical point dried in carbon dioxide for approximately 1.5 hours, followed by immersion in 100% acetone. The sections were mounted with colloidal silver paste onto aluminum stubs and sputter-coated with gold in a Polaron E5000 sputter coating unit (Quorum Technologies, U.K.) at a current of 20 mA for two minutes. Further samples were prepared using the above technique except that they were vapor fixed according to the method of King and Brown (1983). Specimens were examined with a Hitachi S500 Scanning electron microscope (Hitachi Corporation, Tokyo, Japan) at accelerating voltages of between 10 and 25kv.

Transmission light microscopy examination of diseased and healthy tissue samples were prepared by mounting longitudinal xylem sections of approximately 1 cm^3 in glycerin on the stage of a freeze microtome (Hitachi D300) set at -15°C. Once the section was firmly frozen onto the stage, sections between 5 and 20 microns were cut in the plane of the xylem vessels. These sections were immersed in a mixture of 0.1% cotton blue in a 1 to 9 solution of lactophenol in water. The sections were left for 15 minutes in this solution, removed and placed in warm deionized water for a further 10 minutes under gentle agitation. The sections were removed and mounted in glycerol on a microscope slide.

Petri dish cultures of the GWD isolates were examined directly by stereo and transmission light microscopy. Specimen mounts for transmission light microscopic examination of the *N. psidii* isolates in Petri dish PDA cultures were prepared by taking 2 mm wide sections cut perpendicular to the surface of the growth media and mounting the section longitudinally on a

microscope slide in 10% glycerol solution. Mounts were stained with two to three drops of cotton blue and left for 10 minutes at room temperature after which they viewed.

3.2.3   Maintenance of isolates and type cultures, morphological characterization of the causal organism of GWD

All isolates from the various sources and geographical regions (Tables 3.1 and Chapter 2, Fig. 2.1) were single spored and maintained in slant cultures on half strength corn meal agar (CMA, Sparks, Nevada, USA). These slants, as well as slants immersed under oil, were sealed with tobacco paper (Hawksworth, 1974), capped and stored at 8°C and in darkness. Lyophilized cultures were also prepared and stored at 8°C and -6°C. The *N. psidii* isolates were compared to the type cultures (Table 3.2) and descriptions contained in the following keys:

- A Manual of the Penicillia by Raper and Thom, (1949);

- Contribution a l'Etude Morphogenique du Genre *Gliocladium* by Morquer, Viala, Rouch, Fayret and Berge (1963);

- Commonwealth Mycological Institute, Descriptions of Plant Pathogenic Fungi and Bacteria (1964 to present);

- *Cephalosporium*-artige Schimmelpilze (Hyphomycetes) by Gams (1971);

- *Paecilomyces* and some Allied Hyphomycetes by Samson (1974);

- The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces* by Pitt (1979);

- Compendium of Soil Fungi by Domsch, Gams, and Anderson, (1980);

- The Genera of Fungi Sporulating in Pure Culture by von Arx (1981);

- *Penicillium* and *Acremonium* by Peberdy (1987).

Type cultures of the *N. psidii* isolates were sent to mycological institutions for verification of their putative identification (CABI, Bioscience, formally the 'International Mycological Institute'). The *N. psidii* isolate type cultures used in these studies were from South Africa [designated CSF 221 (=IMI 279785 = CBS 912.85)] and Taiwan [designated CSF t226 (= IMI 277770)].

3.2.4 Genomic analysis of the South African type *N. psidii* isolate (CSF 221)

The South African *N. psidii* isolate (CSF 221) was established in axenic submerged liquid culture in ten 250 ml conical flasks each containing 150 ml of Czapek's broth medium. The cultures were incubated under diurnal natural lighting conditions at 30°C for 10 days. At the end of the incubation period, mycelia was collected by coarse filtration (400 mesh filter plastic filter), followed by washing in SDW three times. The mycelium was blotted dry on filter paper, frozen at -70°C and lyophilized and ground in 1.5 ml microcentrifuge tubes with a sterile applicator stick under liquid nitrogen. DNA was extracted utilizing the methods of Rehner and Samuels (1994). The D1-D2 DNA region of the 28S large sub unit and the associated ITS (internal transcribed spacer) regions were amplified and sequenced, utilizing the MicroSeq[®] D2 LSU rDNA fungal identification kit[®] [(Applied Biosciences Inc., Foster City, California, U.S.A), Anonymous, 2011]. Samples were electrophoresed utilizing a ABI Prism 3100 genetic analyzer (Applied Biosciences Inc.) and the sequence data analyzed and compared utilizing MicroSEQ[®] software (Applied Biosciences Inc., Foster City, California, U.S.A). Sequencing results were compared to the GenBank[®] database which is an open access repository for

microbial genetic sequence data (www.ncbi.nlm.nih.gov/BLAST/blast/help.html), which is continually updated (Benson *et al*., 2012). This international database repository is maintained by the US National Institute for Health (Bethesda, Maryland, USA). From these known fungal sequence data, alignment matches were generated and presented as percentage genetic distance as well as a neighborhood relatedness phylogenetic tree (cladogram) as described by Saitou and Nei (1987) and Kurtzman and Robnett (1995). The *N. psidii* DNA sample code used in these studies were C26768 GRE 001and corresponded to the isolate CSF 221 (= IMI 279785).

3.2.5 Type Culture Pathogenicity Tests

Reference and type cultures obtained from various individuals and mycological institutes, as presented in Table 3.2, were sub-cultured on potato dextrose agar [PDA, (Difco, Sparks, Nevada, USA)] in Petri dishes and incubated under conditions as described earlier. After 8 days incubation, a 5mm diameter plug of mycelium was removed from 5 mm behind the advancing front of the expanding colony on PDA. These plugs were inserted into a $2cm^2$ bark flap on guava tree trunks, 10 cm above the soil line, of 1 year old greenhouse grown clonal guavas (cv. Fan Retief), as shown in Chapter 2, Fig. 2.1. The wound was gently wrapped in Parafilm and covered with masking tape. These plants were similarly placed in greenhouse chambers at $25^{\circ C}$ and 33°C and observed for GWD symptoms over 4-5 months. A minimum of five cloned guavas plants were used per treatment, and they were laid out in a completely randomized single tree plot design.

3.3   DATA ANALYSES

Data were analyzed by one or two way analysis of variance and where appropriate, treatment means were separated using the Waller-Duncan k-ratio t test (Little and Hills 1990).

3.4 RESULTS

3.4.1 Taxonomic characteristics of the causal fungi of GWD and TGWD, *N. psidii*

In CMA and PDA cultures, the *N. psidii* isolates most closely resembled the type reference culture of *Gliocladium vermoesenii* (Fig. 3.1 and Table 3.2). The isolates grew at similar rates, and the surface mycelia at seven to ten days in culture were off-white to light salmon pink in color (Figs. 3.1, 3.2 and 3.3), corresponding to Shade 3A, Plate 43 of the Dictionary of Color (Maerz and Paul, 1930). The reverse side of the culture (bottom of Petri dish) was light
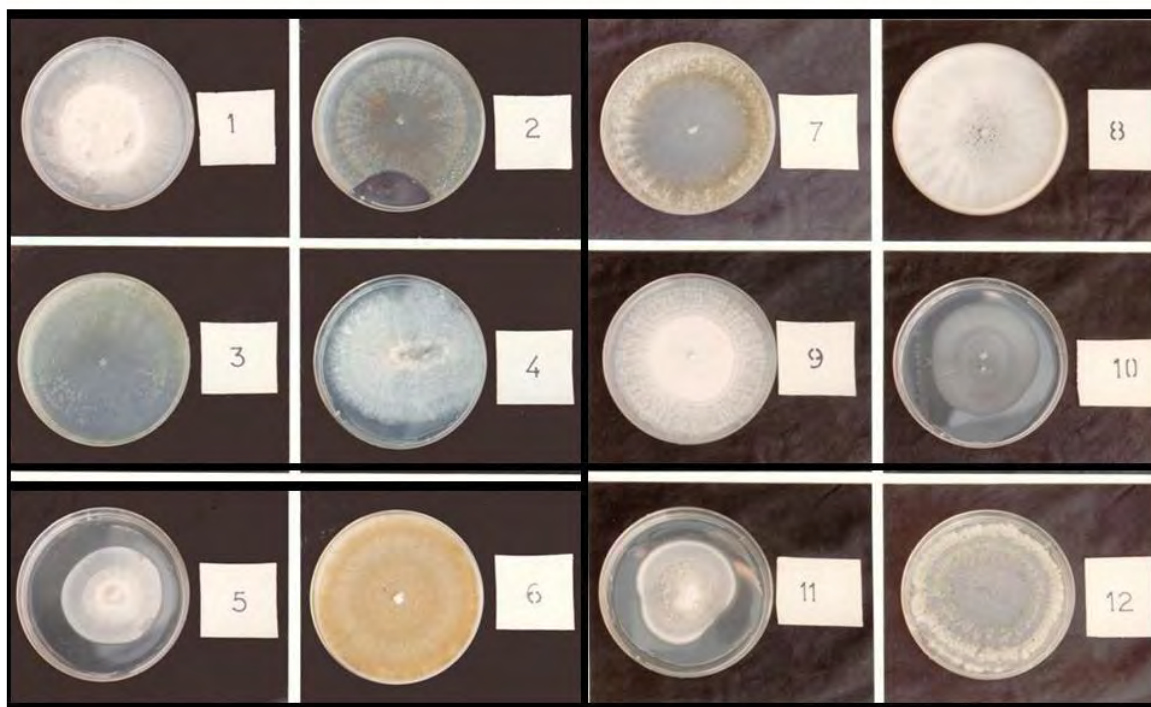
Figure 3.1. Comparative cultural morphology of South African *Nalanthamala psidii* isolates

with reference type cultures:

1. *Gliocladium album;*
2. *G. virens;*
3. *G. viride;*
4. *G. deliquescens;*
5. *G. flavofuscum;*
6. *G. roseum;*
7. *G. catenulatum;*
8. *G. vermoesenii;*
9. *Nalanthamala* sp. (GWD isolate);
10. *Paecilomyces* spp.;
11. *Acremonium diospyri;*
12. *Nalanthamala* sp. (GWD isolate).

orange/yellow (Fig. 3.2), corresponding to Shade 1D, Plate 45 of the Dictionary of Color

(Maerz and Paul, 1930). Both *G. vermoesenii*, and the *N. psidii* isolates exhibited slightly

flocculose mycelium that was radially stranded, giving rise to diurnal ring patterns when grown

under a 12 hour day / 12 night lighting regime (Figs. 3.1, 3.2 and 3.3). Viewed microscopically,

the cultures of *N. psidii* from South Africa and Taiwan were identical.
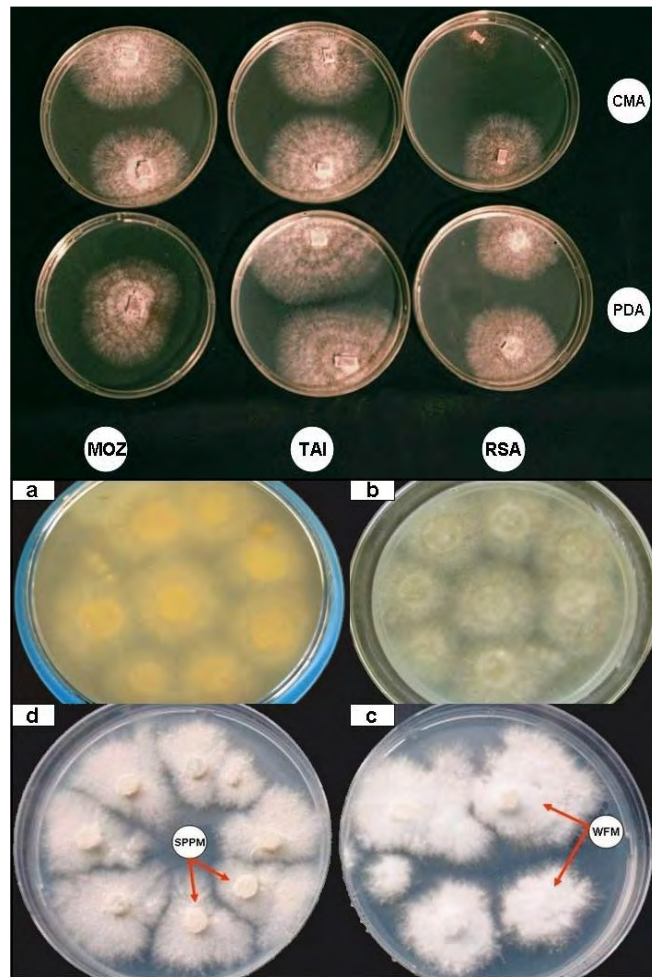
Figure 3.2. Cultural characteristics of the GWD organisms: Top photograph: Comparative cultural morphology of various *Nalanthamala psidii*, single spore isolates.
MOZ = Mozambique isolate (CSF M3);
TAI = Republic of China, Taiwan (CSF t226);
RSA = Republic of South Africa (CSF 202).
The top cultures are grown on corn meal agar, the bottom cultures on PDA.
Bottom photograph:
a, posterior view of a 10 day old culture exhibiting typical yellow pigmentation;
b, typical white floccose mycelial growth of a 10 day old non-pathogenic culture on CMA;
c, typical white floccose mycelial growth of a 7 day old non-pathogenic culture on PDA;
d. pathogenic 7 day old PDA culture.
SPPM = Striate, pink, prostrate mycelia. WFM = White, floccose mycelia.
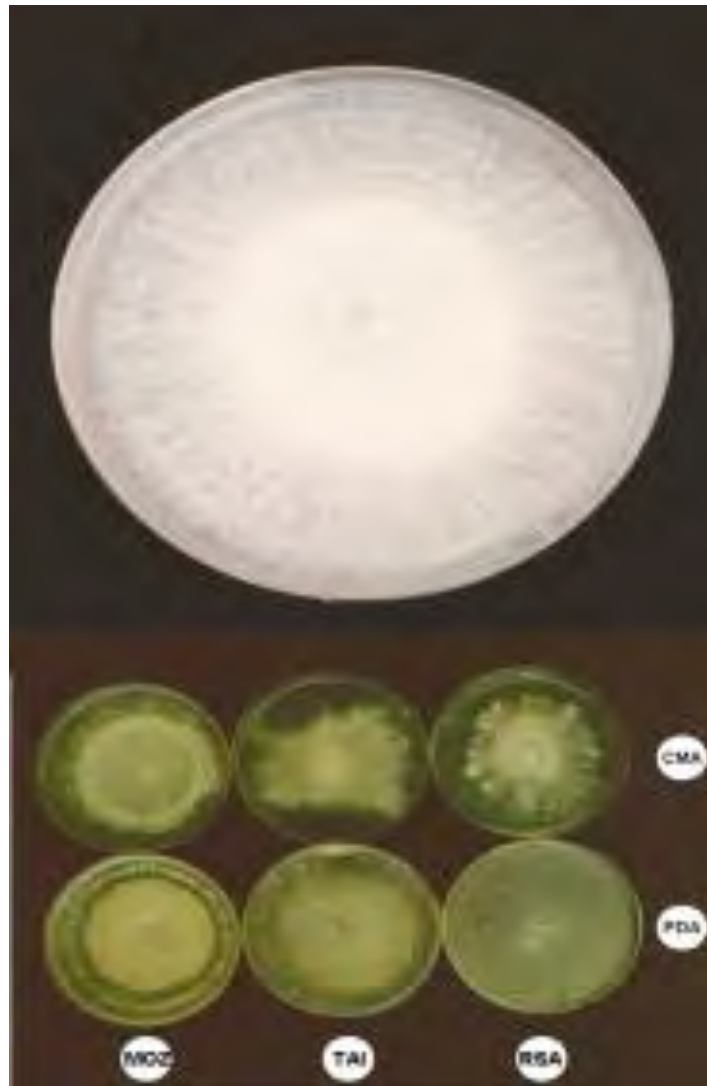
Figure 3.3. Cultural characteristics of the GWD organisms:

Top photograph: cultural diurnal radial growth patterns in pathogenic *N. psidii* isolates grown for 10 days on PDA under 12 hours of incandescent light illumination per day. Bottom photograph; Non-pathogenic strains of the *N. psidii* isolates from Southern Africa generated through repeated sub culturing. Cultures were grown under 12 h incandescent light cycles at 30°C. Note mycelia is largely white and floccose with a general lack of diurnal rings. MOZ = Mozambique isolate (CSF M1); TAI = Republic of China, Taiwan (CSF t201); RSA = Republic of South Africa (CSF 218).

Single sporing of these isolates resulted in colonies with similar macroscopic cultural

morphology which appeared in culture to be vegetatively compatible (Fig. 3.2). Conidial clones of the type cultures also showed little or no variation. Type cultures of *Septofusidium elegantulum* (Pidopl.) W. Gams and *Nalanthamala madreeya* Subramanian were not available, so comparisons with these fungi were made on the basis of previous descriptions (Subramanian, 1956; Gams, 1971).

Both fungi produced two morphologically distinct types of hyphae, as did *G. vermoesenii* (Fig. 3.4). The first type was broader (2.5-3.5 μm) and was abundant in fresh cultures (under seven days old). The second type of hyphae was narrower (0.7-1.2 μm) and arose from the thicker hyphae at a septum, or by simple branching. The thinner hyphae were often observed in older cultures (approximately one month old) arising from a septum bordering a degenerating portion of the hyphae, and was often found growing endogenously through it, later emerging through the decaying hyphal cell wall.
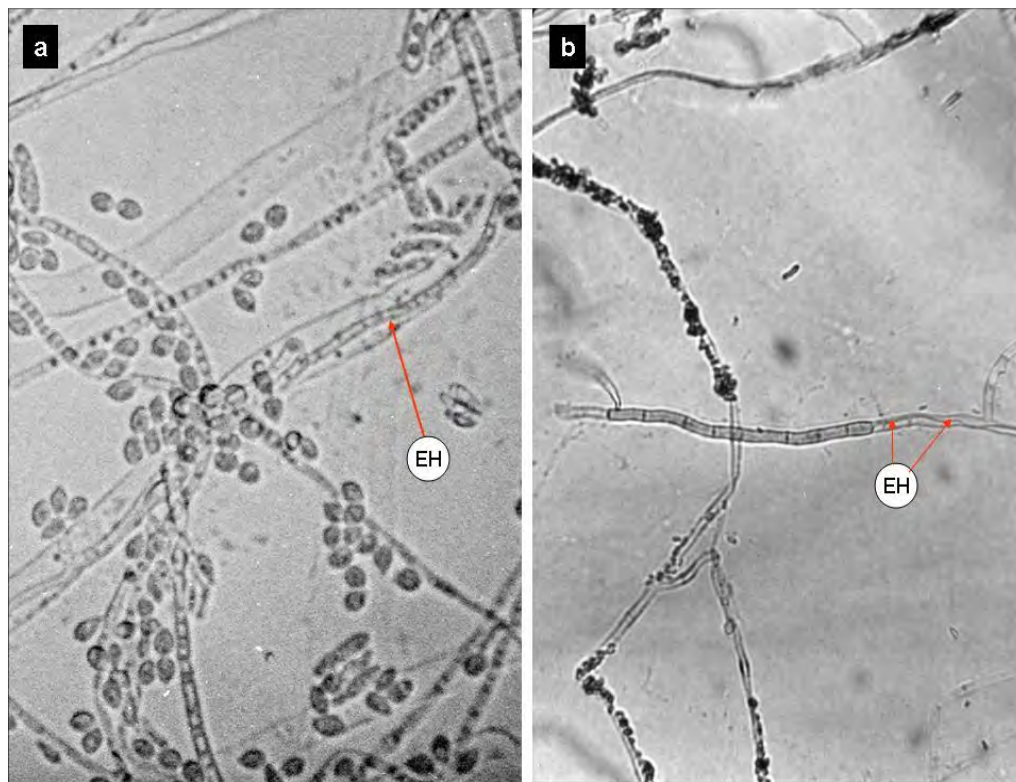
Figure 3.4 Endogenous hyphae (EH) in a *N. psidii* isolate (a) and a type culture (IMI 40231) of *G. vermoesenii* (b). Both grown on CMA for 10 days in darkness.

This type of narrow hyphae was also observed penetrating xylem wall pits of guava plants (Figs. 3.5 and 3.6). The narrow hyphae observed *in situ* has an approximate width of 1.5-2.0 μm compared to the thicker hyphae which had an approximate width of 3.5-4.5 μm (Fig. 3.7). The xylem pits of guava are approximately 2-3 μm across and 20-30 μm in length (Fig 3.7) but are usually occluded with an amorphous aggregate, resulting in an aperture of 1.0-1.5 μm. The *G. vermoesenii* type isolate was also observed to produce endogenous hyphae (Fig. 3.4), whereas none of the other type reference cultures examined exhibited this phenomenon, including other species of *Gliocladium*.
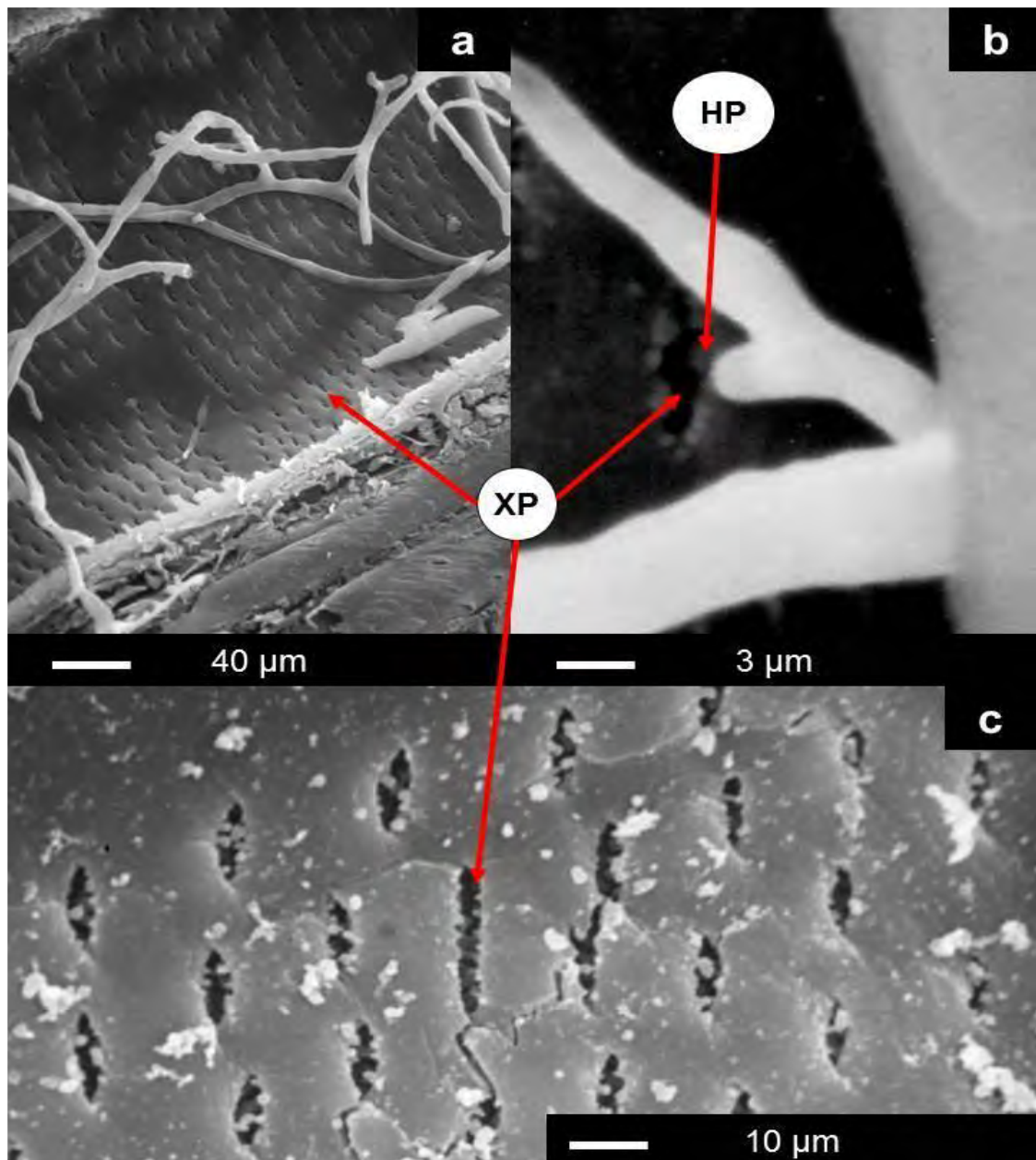
Figure 3.5 Scanning electron micrographs of xylem vessels from a GWD symptomatic tree:
a, colonization of a xylem vessel by fungal mycelia;
b, commencement of fungal penetration of a xylem pit;
c, enlarged view of xylem pits in the wall of a xylem vessel.
XP = xylem pit. HP = xylem pit penetrating hypha.
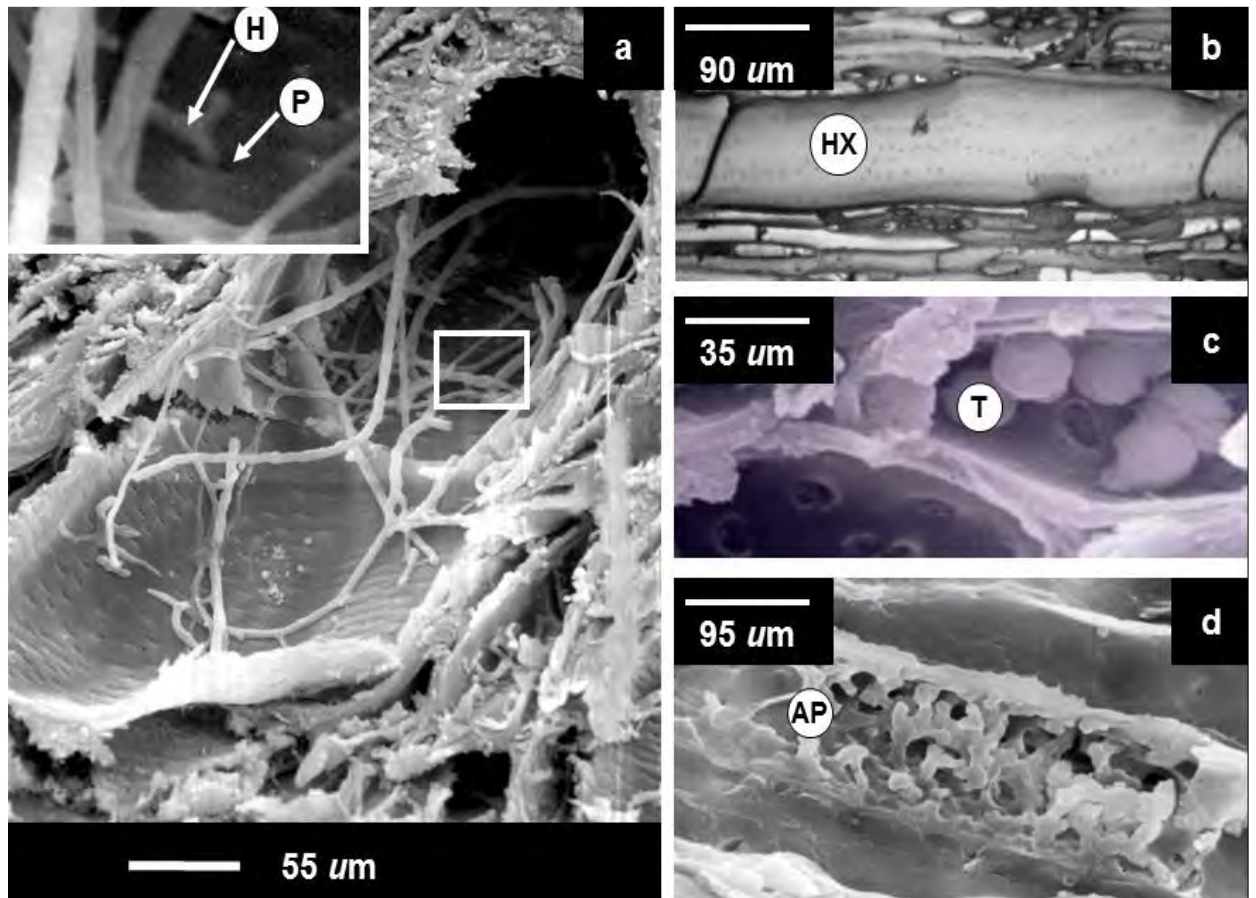
Figure 3.6. Scanning electron micrographs of xylem vessels in healthy and diseased guavas:
a, xylem vessel from a guava tree infected with *N. psidii* showing hyphal penetration of xylem pits (inset is an enlarged section of the demarcated area);
a. xylem in a healthy guava tree;
b, tyloses in a guava xylem vessel in advance of hyphal colonization in diseased guava scaffold branch tissues;
c, amorphous plugs in the xylem vessels in primary root tissue of infected guavas.
H= hypha. P = Xylem pit. HX= Healthy xylem. T=Tyloses. AP=amorphous plugs.
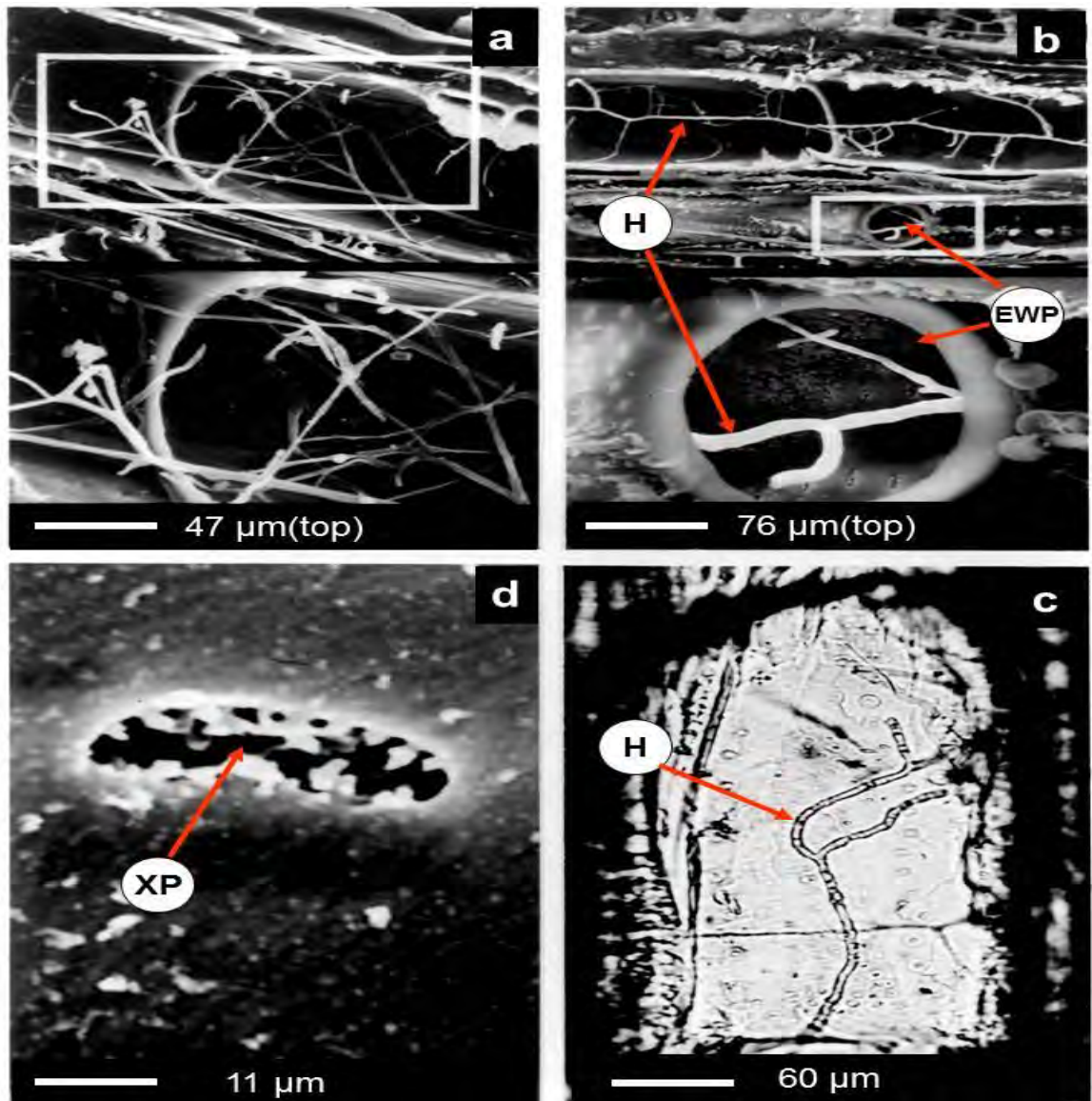
Figure 3.7. Scanning electron and transmission light micrographs of xylem vessels from a GWD infected tree:

a and b. scanning electron micrograph of longitudinal sections of xylem vessels colonized by fungal hyphae (H);

c. transmission light micrograph of fungal hyphae (H) colonizing a guava xylem vessel from a GWD symptomatic tree;

d. scanning electron micrograph of a xylem wall pit (XP) with amorphous matter emanating from the inner wall surface and partially occluding the pit orifice.

EWP = Xylem vessel terminal end wall portal.

Conidiophores were borne on both types of hyphae and were of two types (Figs. 3.8, 3.9 and 3.10). The first type was generally erect, smooth, penicillate and produced dry, catenate conidia, enteroblastically (Type 1 conidia). The conidiophores were observed to produce up to three branches from which up to seven metulae arose, generally producing a whorl of cylindrical, slightly divergent conidiogenous cells, tapering to a short collula, with small, limited collerettes (Figs. 3.8, 3.9 and 3.10).

Conidiogenous cells were observed to have linear dimensions of 7-25 x 2.5-4.0 μm. The conidia (Figs. 3.9, 3.10 and 3.11) produced from these conidiophores were smooth, ellipsoidal to limoniform in shape, and were hydrophobic.

The conidia were observed to have distinct disjunctors between them (Figs. 3.9 and 3.10). Their mean dimensions were (2.5-4.5) x (1.5-2.5) μm.
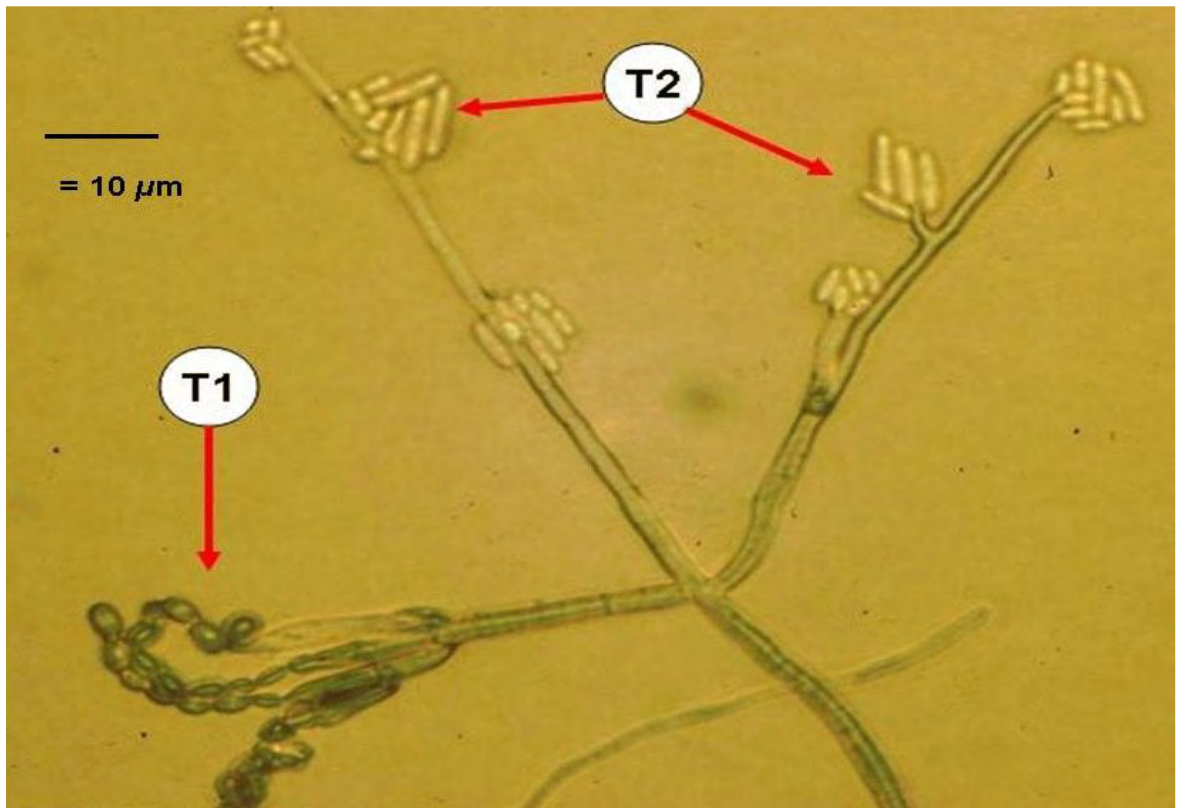
Figure 3.8 Conidia of *Nalanthamala psidii*. Type 1 (T1) and Type 2 (T2) conidia emanating from the same hypha of a *N. psidii* culture grown on PDA for 7 days.
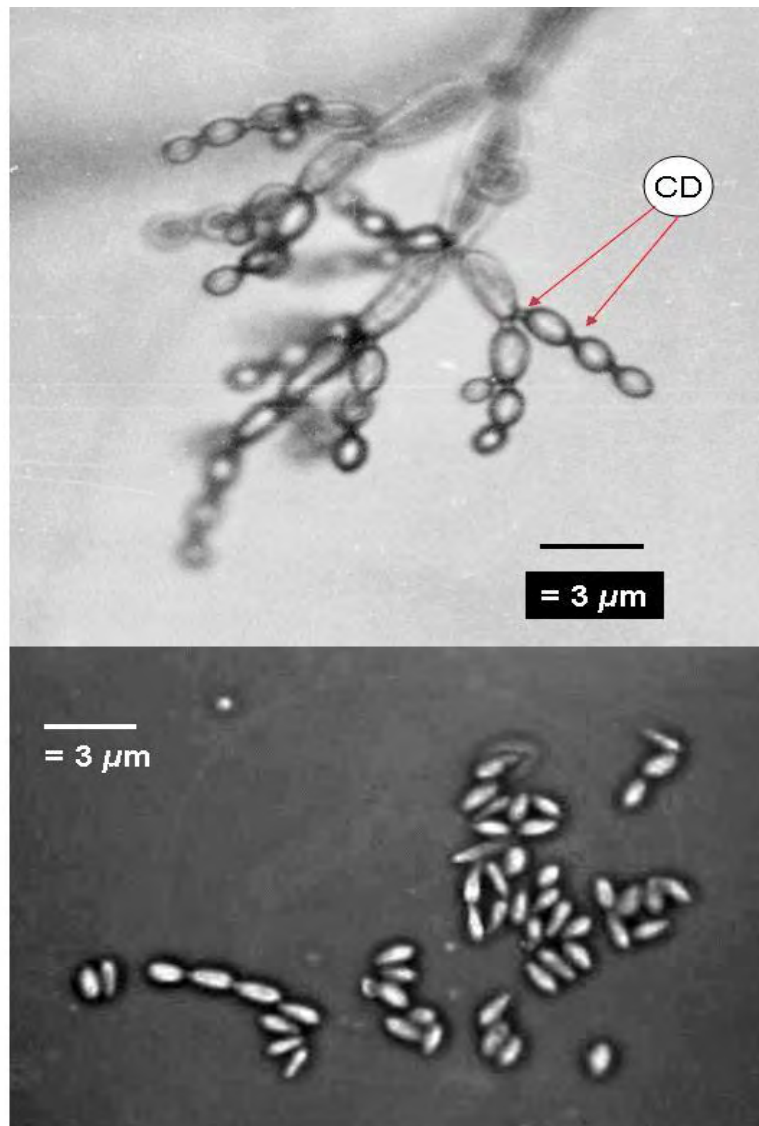
Figure 3.9. Conidiophores of *Nalanthamala psidii*:
Top: conidiophore with Type 1 chains of conidia with conidial disjunctors (CD);
Bottom: Type 1 conidia separated from the conidiophore in chains and released (bottom).
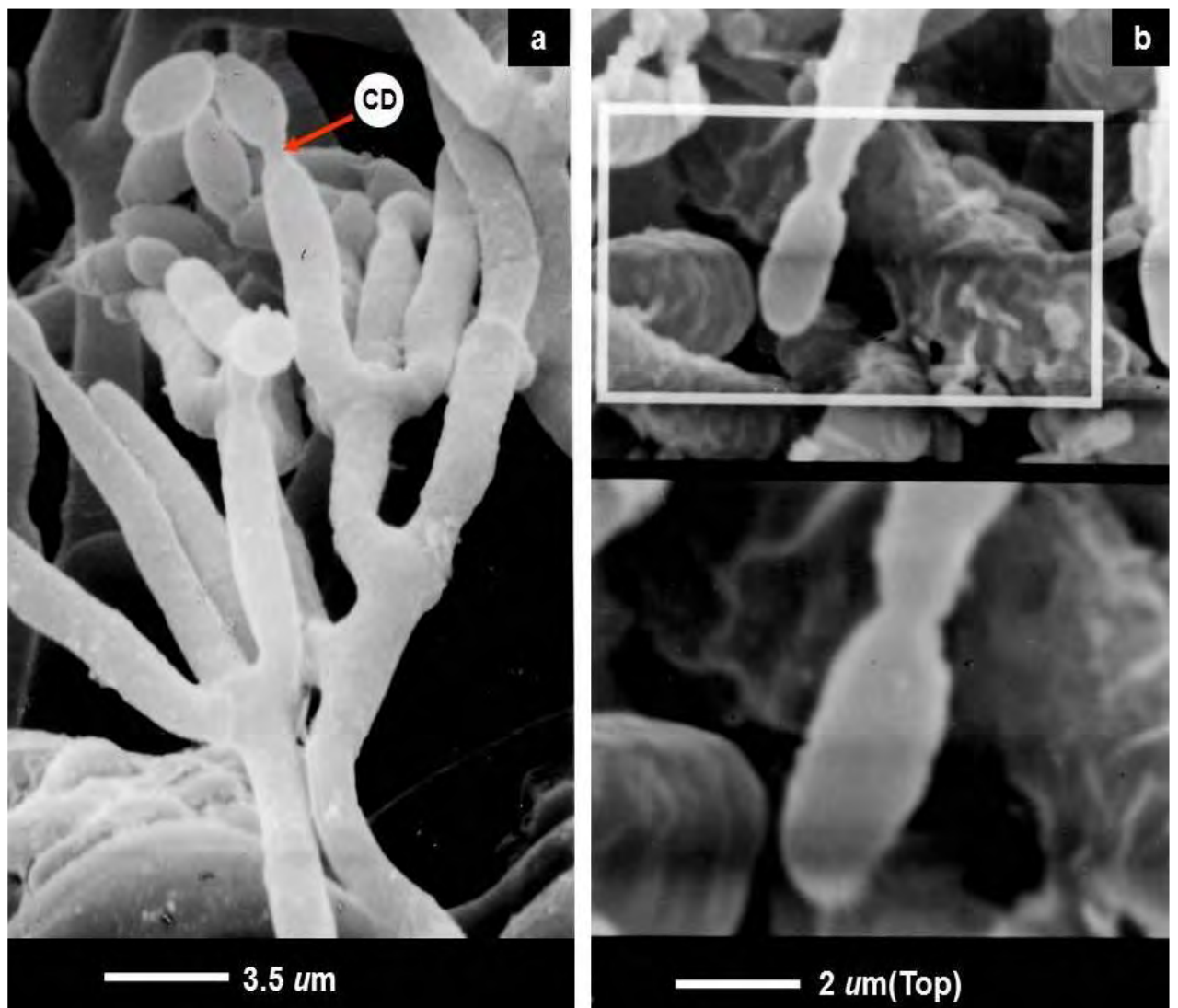
Figure 3.10. Conidiogenesis of *Nalanthamala psidii*:
Left: Type 1 catenate conidiogenesis on penicillate conidiophores with conidial disjunctors (CD) visible (a);
Right: Type 2 conidiogenesis on a solitary conidiophore (b).

The second type of conidium (Type 2 conidia, Figs. 3.8, 3.10, 3.11 and 3.12) is typically larger (5.5-16 x 1.5-3.0 μm), hydrophilic, aseptate, frequently bi-guttulate, ellipsoidal to nephriform in shape, produced enteroblastically on solitary, smooth conidiophores, which develop terminally and intercalary (Figs. 3.10 and 3.12), with dimensions of 15-30 x 2.5-4 μm. These conidia where observed to aggregate in mucilage that forms a solitary gelatinous head (Fig.3.8). These conidia were produced in culture more abundantly from juvenile hyphae, as opposed to the Type 1 conidia that arose more abundantly from older mycelium (3-5 days). Type 2 conidia germinated from their polar regions (Fig. 3.12).
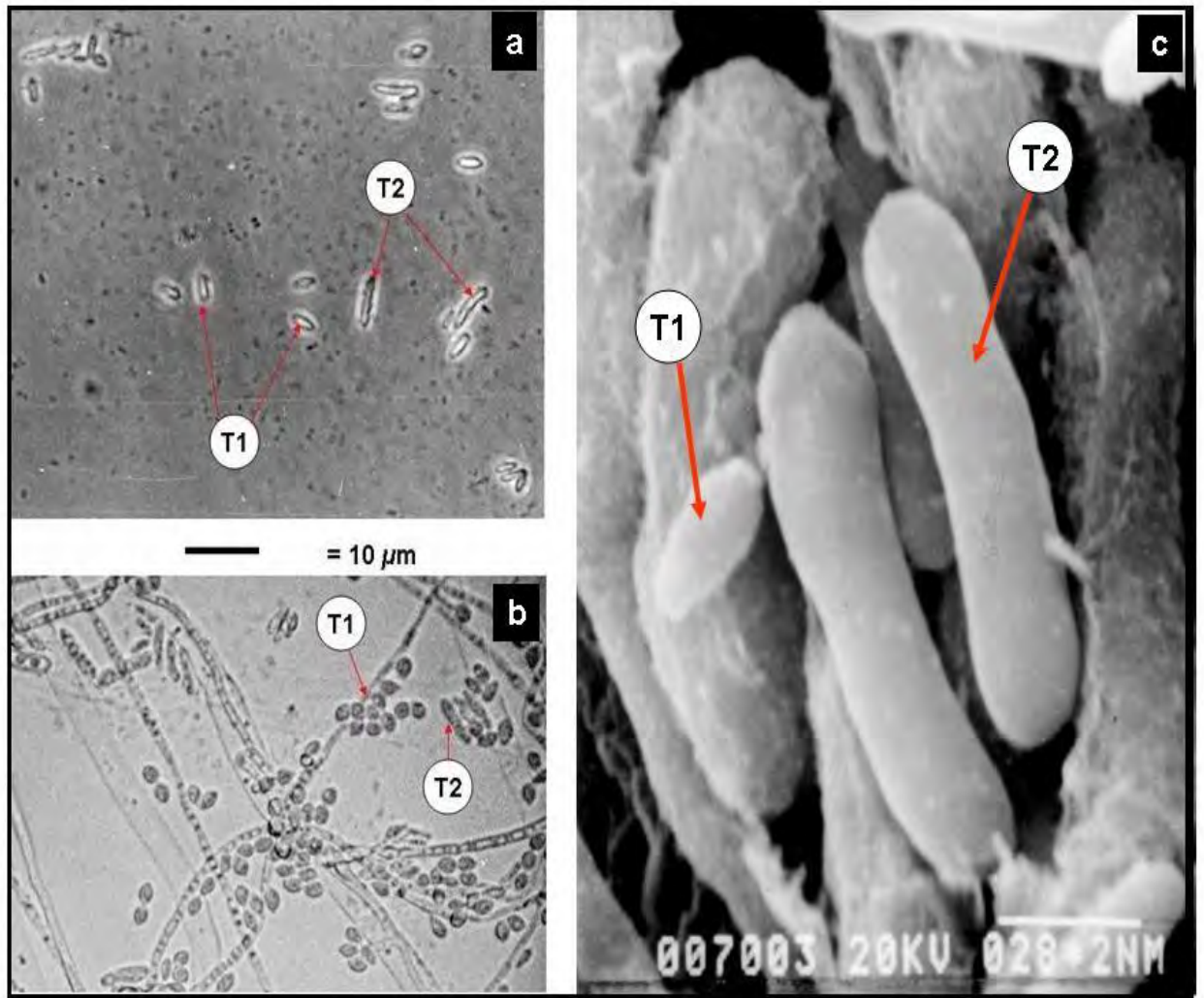
Figure 3.11. Conidia of *Nalanthamala psidii*. Type 1 (T1) and Type 2 (T2) conidia of *N. psidii* grown on PDA for 10 days: Left: a and b. transmission light microscopy of Type 1 and Type 2 conidia; Right: c. Scanning electron microscopy of both types of conidia of *N. psidii*.
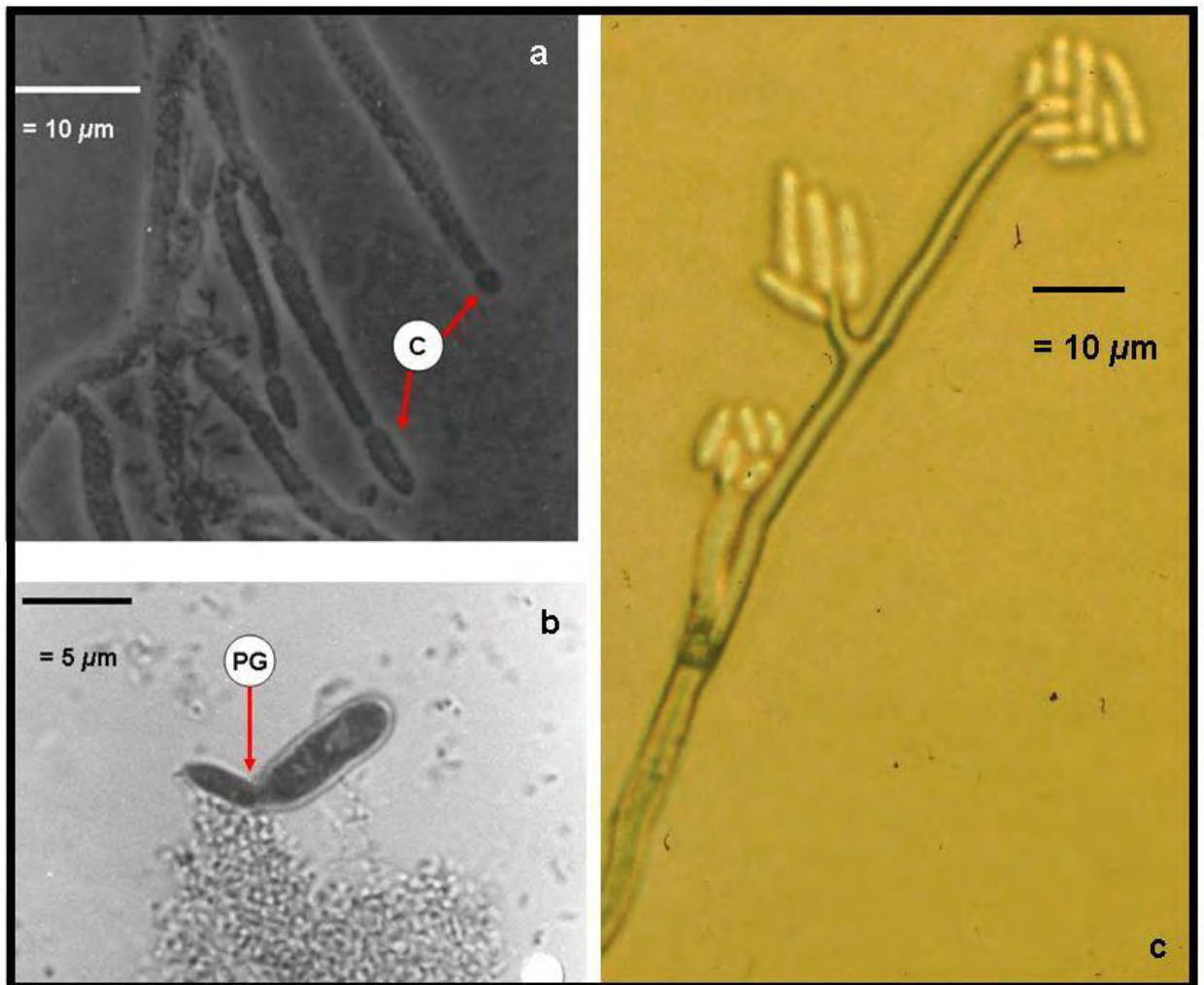
Figure 3.12. Conidiogenesis of *Nalanthamala psidii*:
Left: a. Type 2 conidiogenesis;
          b. Germinating Type 2 conidium.
Right: c. Type 2 conidial formation in slime heads on solitary terminal or lateral conidiophores.
PG = Posterior germ tube emergence.

Chlamydospores were also produced (Fig. 3.13) when the fungus was grown either on solid media, or (more abundantly) in a submerged liquid culture medium (Czapek's broth medium). Chlamydospores are observed more often on older cultures, grown on a wide variety of agar media. The chlamydospores were found to be terminal or intercalary, hyaline, thick-walled and unicellular (Fig. 3.13).
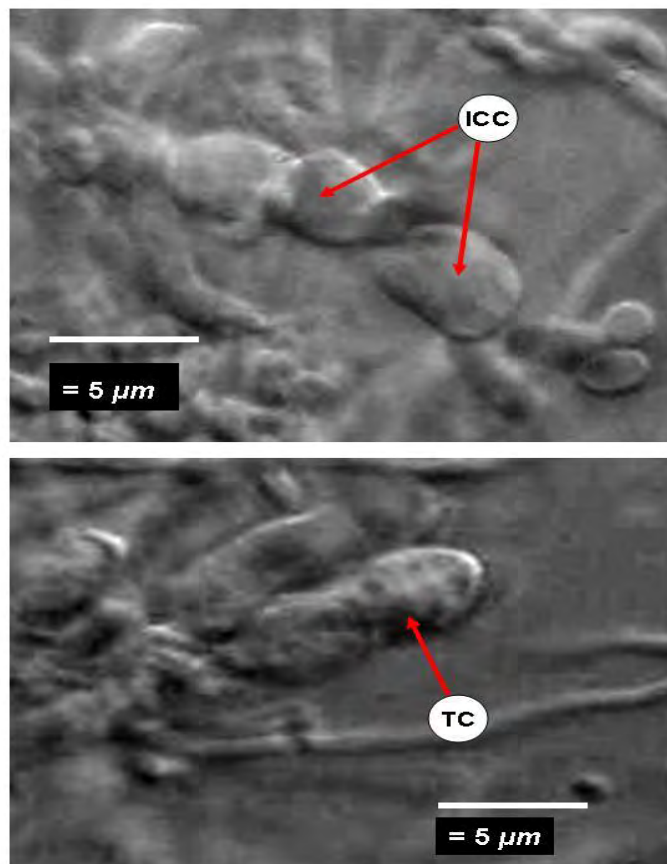
Figure 3.13. Formation of chlamydospores by *Nalanthamala psidii*:
Hyphae of a *N. psidii* isolate from submerged cultures grown for 20 days in Czapek's Dox broth.
Top: Intercalary chlamydospore (ICC).
Bottom: Terminal chlamydospore (TC).

When the *N. psidii* was grown in submerged cultures it retained an off-white to beige color, whereas the *G. vermoesenii* type cultures became dark green in submerged culture. When grown in darkness, *N. psidii* generally did not develop any gross cultural coloration.

With the exception of *G. vermoesenii*, the other type reference cultures did not produced two conidial forms (Tables 3.3 and 3.4). However, *G. vermoesenii* mycelia in submerged culture (Czapek's broth) developed a distinctive green hue, whereas the *N. psidii* isolates did not. The conidial dimensions of the CSF 221 and CSF t226 isolates were generally different from any of the other type reference cultures (Table 3.3).

Table 3.3. Taxonomic Comparisons with Certain Closely Related Fungi to the *Nalanthamala psidii* Isolates

Conidial dimensions

Isolate[a]	Length (μm)[b]				Width (μm)[b]		
	Range	Mean	sd[c]		Range	mean	sd[c]
GWD							
CSF 221							
Type 1	2.5-4.5	3.2	0.3		1.5-2.5	1.8	0.2
Type 2	5.5-16	8.6	1.8		1.5-3.0	2.0	0.4
TGWD							
CSFt226							
Type 1	2.5-4	3.0	0.2		1.5-2.5	1.8	0.2
Type 2	4-11	7.5	1.5		1.5-3.0	2.2	0.5
A. diospyri	3.5-5	4.2	0.5		2-2.5	2.2	0.3
G. vermoesenii							
Type 1	2-4.5	3.5	0.2		1.5-3	2.1	0.2
Type 2	4-9	6	1.4		2-3.5	2.4	0.2
G. roseum							
Type 1	3-5	4	0.3		3-4.5	3.5	0.2
Type 2	6.5-9	7.6	1.4		3-4	3.2	0.3
G. caten-ulatum	4-7	5.5	0.3		3-4.5	3.5	0.2

[a] Isolates that produced two types of spore are indicated by a second row of dimensions.
[b] Two hundred conidia per isolate were measured.
[c] sd= standard deviation

Cultures of the causal fungi of GWD or TGWD maintained on half strength CMA (with or without oil immersion) and bi-annually sub-cultured onto autoclaved guava wood agar (water agar with small pieces of 1-2 year old guava wood shavings on the agar surface) maintained their viability and pathogenicity for at least 1-2 years. Lyophilized agar cultures also remained viable for at least a period of seven years. However, if the *N. psidii* isolates were sub-cultured

repeatedly and maintained on rich media such as PDA, all isolates tested underwent a change in cultural morphology within 5-10 transfers on PDA, particularly at higher incubation temperatures. Mycelia became floccose, effuse and aerial (Fig. 3.2). Conidial production was diminished (particularly Type 1 conidia) and the color of the culture became an off white to light cream. The reverse (posterior) color of the culture remained a light yellow color after 7-10 days in culture. Once *N. psidii* isolates had undergone this cultural metamorphosis, they expressed a diminished pathogenicity, or in some cases, they were completely non-pathogenic (Fig. 3.2).

3.4.2   Genomic analysis of the South African type GWD isolate (CSF 221) of *N. psidii*

The guava wilt pathogen's DNA sequence analysis of the D2 region of the 28S large subunit genome, indicated that the pathogen most closely matched (99% homology) the sequence analysis of *Nalanthamala psidii*, which is closely related to *Nectria cinnabarina* (Fig. 3.14). The pathogens causing GWD and TGWD can be placed within the taxa of the Nectriaceae (Schroers *et al*., 2005).

Neighbor JoiningTree[a]

```
N Join:  4.900 %  ─────────────────────────────
                        ┌── Sphaerostilbella novaezelandiae
                        │   ┌── Paecilomyces viridis
                        │   │  ┌ Epichloe clarkii
                        │   │  └ Epichloe typhina
                        │   ├── Stilbella jaapii
                        │   │  ┌ Fusarium aquaeductuum var. aquaeductuum
                        │   │  └ Fusarium aquaeductuum var. aquaeductuum
                        │  ┌─── C26768 GRE 001[b]
                        │  ├── Nectria cinnabarina
                        │  └── Nectria flavolanata
            └── Nectria macrostoma
```

Alignment: C26768 GRE 001[b]
  5.06 % 316 Nectria cinnabarina
  5.35 % 318 Fusarium aquaeductuum var. aquaeductuum
  5.35 % 318 Fusarium aquaeductuum var. aquaeductuum
  6.29 % 319 Epichloe clarkii
  6.29 % 319 Stilbella jaapii
  6.29 % 319 Epichloe typhina
  6.92 % 318 Nectria flavolanata
  6.92 % 319 Paecilomyces viridis
  6.92 % 319 Sphaerostilbella novaezelandiae
  6.94 % 317 Nectria macrostoma

Fig. 3.14. Fungal DNA sequence analysis of the 28S (large sub unit) D2 region of the rRNA genome of the guava wilt pathogen and its corresponding placement in a neighbor joining phylogram of similarly sequenced fungi.

[a] Sequence alignment as compared to known fungal sequence analysis of the 28S (large sub unit) D2 region of the rRNA genome GenBank® database. Alignment matches are presented as a percent genetic distance format. Low percent indicates a close match.

[b] C26768 GRE 001 is CSF 221(= IMI 279785). Closest sequence homology indicates that the GWD pathogen aligns with *Nalanthamala psidii* at >99% correlation.
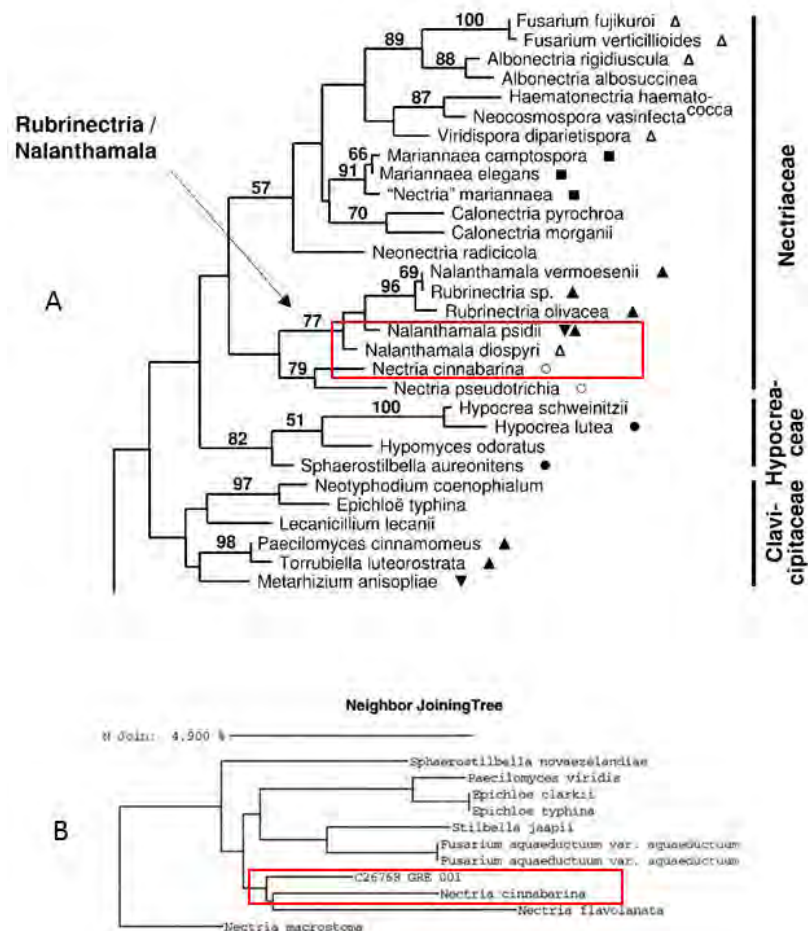
Fig. 3.15. Comparison of the parsimonious phylograms from independent LSU rDNA sequence data from the GWD pathogen.

Top (A) from Schroers *et al*., 2005.

Bottom (B) current study.

Both data sets indicate that the GWD pathogen aligns with *Nalanthamala psidii* with a >99% correlation.

3.4.3 Pathogenicity tests

Plant mortality was only observed where the GWD and TGWD *N. psidii* isolates (conidia or mycelia) were wound inoculated into the guava tree clones (Chapter 2, Table 2.2 and Chapter 4, Fig 4.5). Fungal colonization of the xylem vessels was observed to occur significantly faster acropetally than basipetally (Chapter 2, Fig. 2.3). In most of the inoculated trees, at the time of tree death, the GWD and TGWD *N. psidii* isolates were recovered in lower frequencies from the xylem tissues below the trunk inoculation point as compared to recoveries from above the inoculation point. Unwounded trees inoculated with the GWD or TGWD *N. psidii* isolates (conidia or mycelia) remained healthy over the course of the experiment (Chapter 2, Table 2.2). None of the trees inoculated (wounded) with the type reference cultures died (Table 3.2). However, *Gliocladium vermoesenii*, *G. viride* and *Acremonium diospyri* were recovered from their inoculation sites, although tissue necrosis was absent. All the control trees survived. Wound inoculation with CSF 207 (a low pathogenicity strain due to repeated sub-culturing) did not result in any plant mortality over 90 days. However, at 120 days post inoculation, two plants expressed GWD symptoms and by 150 days, these plants died. The GWD pathogen was re-isolated and the cultural morphology of the isolate was identical to the pathogenic type culture CSF 221, *N. psidii*. As reported in Chapter 2, when the re-isolated CSF 207 was again inoculated into guava, plant mortality rates were similar to CSF 221.

Gliocladium-like isolates, morphologically identical to the GWD or TGWD *N. psidii* isolates used to inoculate the wounded guavas, were re-isolated from tissue sections taken from dead plants. Tissue isolations from unwounded plants did not result in any fungi being consistently

isolated. Sections of guava wood from diseased or recently dead trees viewed microscopically showed the presence of extensive colonization by fungal mycelia in the active xylem vessels (Figs. 3.5, 3.6 and 3.7). Tyloses were only observed in xylem vessels from above ground tree tissues (Fig. 3.6). Amorphous plugging was observed in xylem vessels only from primary root tissue sections (Fig. 3.6).

The two types of conidia derived from single spored isolates of the TGWD and GWD fungi were found to be equally infective (Chapter 2, Table 2.2). All of the guava plants died within four months when they were wound inoculated with conidia or mycelia from *N. psidii* strain CSF 221 (Chapter 4, Fig.4.5).

Techniques employed to separate the two conidial types resulted in moderate success in as much that a 95% purity level was obtained for the Type 1 conidial type and 80% for the Type 2 conidial type, without loss of viability. Further purification of the Type 2 conidial suspension increased the purity to approximately 90%, but the viability diminished to less than 25% and bacterial contamination increased. Mixed populations of conidia were found to be easily distinguishable by direct microscopic examination of the media surface of Petri dishes on which the conidia were sown, allowing for accurate germination assessments.

3.5   DISCUSSION

3.5.1 Morphological Taxonomic Studies

A new disease of guava, guava wilt disease (GWD), was discovered in Southern Africa in the 1980's. It was the first such report of this disease from Africa (Grech, 1983). A fungus belonging to the genus *Gliocladium* was initially identified as the causal fungus of this disease in these as well as other studies (Vos *et al.*, 1998).

The initial placement of the causal fungus of GWD in the genus *Gliocladium* was in part because all members of the genus produce conidia that are enveloped in a mucilaginous slime head (Morquer *et al.*, 1963; Pitt, 1979; Von Arx, 1981). Conidia are also formed from a complex penicillus, which is also consistent with the *Gliocladia* (Domsch *et al.*, 1980). The *N. psidii* isolates do share characteristics with such genera as *Acremonium*, *Paecilomyces* and *Septofusidium* but fundamental morphological differences, particularly in conidiophore and conidial architecture, exclude their inclusion in these genera. Anomalies in the putative identifications allocated to this fungus over the past several decades are given in Table 3.1. The *N. psidii* isolates are characterized by their ability to produce two morphologically different, aseptate conidial types on conidiogenous cells devoid of a pronounced neck, as well as chlamydospores, a feature not known in *Septofusidium* (Gams, 1971), but known in several species of *Acremonium* (Lin *et al.,* 2004). On the other hand, *Paecilomyces* is monoconidial and thus produces only one type of conidium that shares similarities with the Type 1, dry conidia produced by the GWD pathogens. The *N. psidii* isolates produce Type 1 conidia in dry chains by basipetal succession on a complex penicillus and are catenate. Furthermore, the

conidial chains of *Paecilomyces* differ from the *N. psidii* isolates in that connectives between the conidia are very pronounced, and that the phialides possess a long tapering collula (Samson, 1974). The phialides of the *N. psidii* isolates also do not have a pronounced swollen base (Figs. 3.8 and 3.10), a character possessed by both *Paecilomyces* and, to a lesser extent, *Acremonium* (Barron, 1968; Domsch *et al.*, 1980). Hyphae (often forming hyphal ropes) bearing conidiophores giving rise to simple whorls of phialides of which the base is often found to be covered with wart-like chromophillic encrustations are common in *Acremonium* (Domsch *et al.*, 1980). These morphological features were not observed in the *N. psidii* isolates (Figs. 3.8 and 3.10). Some species of *Acremonium* are known to produce two conidial types, either in dry chains or in slimy heads (Gams, 1971; Domsch *et al.*, 1980). These conidia are septate or aseptate, generally produced on verticillate conidiophores with simple awl-shaped phialides (Domsch *et al.*, 1980) and generally devoid of connectives. These features, as well as the conidiophores of *Acremonium* being not strongly branched, differentiate the *N. psidii* isolates from this genus. Verticillate conidiophores were rarely observed being produced by the *N. psidii* isolates.

Conidiogenesis on host tissues was similarly characterized by dimorphic conidial production. Type 1 conidia are typically produced in what appear to be structures morphologically identical to a sporodochium [a conidial mass supported by a superficial, cushion-like (pulvinate) mass of short conidiophores near the surface of the substrate], which in this case is beneath an integument of host tissue (Hawksworth *et al.*, 1985)]. Typical sporodochial structures produced by the GWD pathogen are shown in Fig. 3.16.
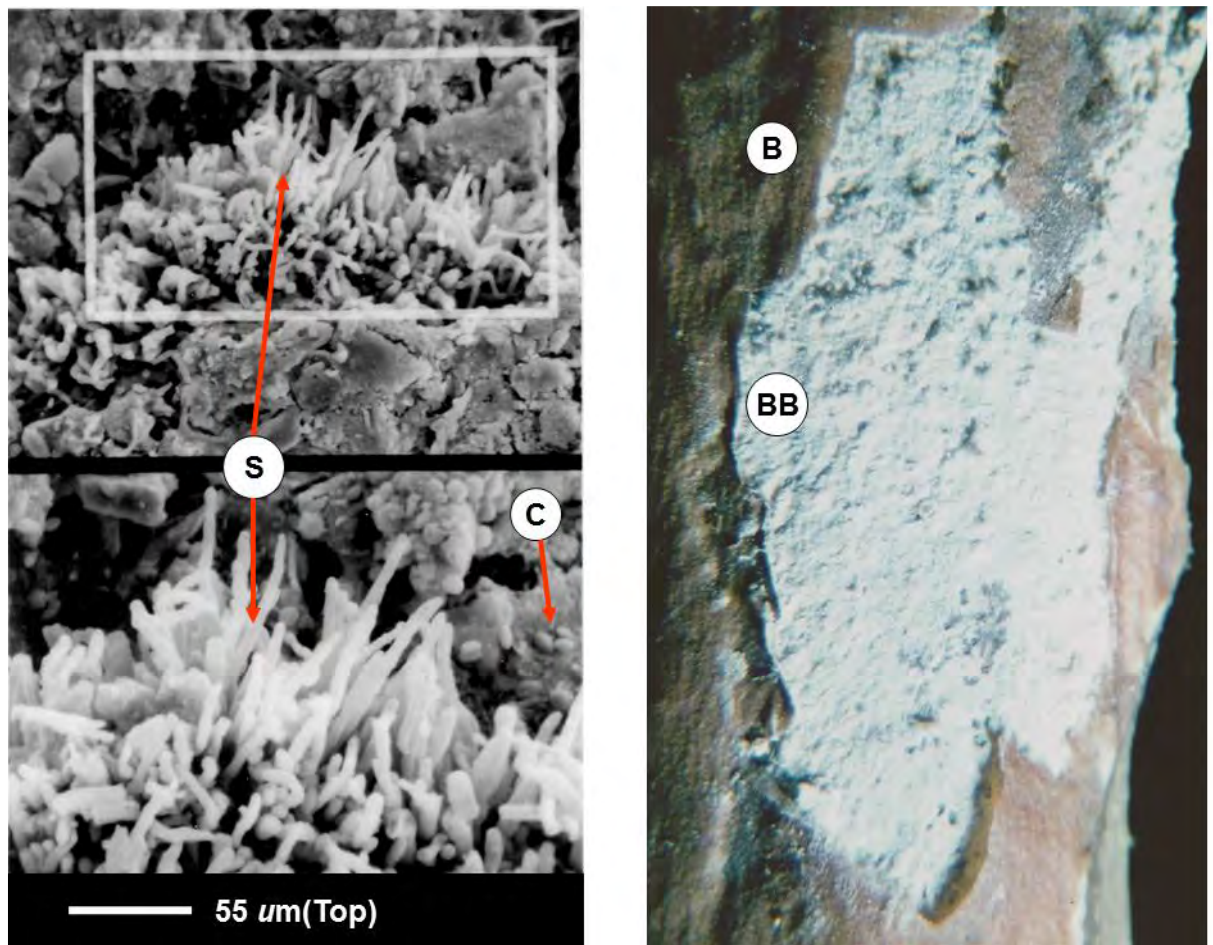
Figure. 3.16. Sporodochial structures of *Nalanthamala psidii*:

Left: scanning electron micrograph showing sporodochial masses (S) and conidia (C);
Right: ruptured bark blisters (BB) on the bark (B) surface of a recently dead guava tree (3 days *post mortem*). The white masses are hyphae and conidia in sporodochial structures.

The causal fungus of persimmon wilt (*Nalanthamala diospyri* = *Acremonium diospyri* = *Cephalosporium diospyri*), has been described as "*producing pink spore masses beneath the bark of wilt killed trees*" (McRitchie, 1979), which is precisely what is observed with trees killed by GWD. Leu and Kao, (1979) named the causal organism of TGWD as *Myxosporium psidii* and described it as producing acervuli. However, acervuli are typical of the Melanconiales but they are not typical of the Moniliales in which *Gliocladium*, *Penicillium*, *Acremonium* and *Nalanthamala* are all placed. Therefore the sporulating structures produced on host tissue by the GWD pathogen are more correctly described taxonomically as sporodochia.

Overall conidial dimensions (Table 3.3), colony morphology, growth habit as well as the above fundamental taxonomic differences, separated the *N. psidii* isolates from these similar genera. To date, endogenous hyphae have not been recorded in any of the compared genera, except *Gliocladium*. Endogenous hyphae have also been recorded from several species including the vascular pathogen *Ceratocystis ulmi* (Buism.) C. Moreau (Kendrick and Molnar 1965), where their presence has been associated with unfavorable environmental conditions for fungal growth, particularly in plant tissues such as the xylem vessels (Urbash 1984). Interestingly, the optimal pH for growth of the *N. psidii* isolates in culture was pH 6.0 to 6.5, which encompasses the pH of guava xylem sap (pH 6.2, Grech unpublished). The discovery that *N. psidii* produces two distinct types of hyphae, observed in culture and *in planta*, in guava xylem vessels (Figs. 3.4-3.7) is highly significant and an important discovery in terms of the etiology and histopathology of this disease. The two hyphal types are distinguishable primarily by their width (Figs. 3.4 and 3.7), Xylem pit ingress exclusively involved the attenuated and slender

hypha (Fig. 3.5), which is approximately 30% of the width of the broader hypha, which usually is restricted to the xylem vessel cavity. This type of hypha may also be an important evolutionary adaption that enables this pathogen to breach xylem pits that typically exclude large diameter hyphae. The slender hyphae were observed arising from septa (Fig.3.4) within larger hyphae and progressing through the degenerating wider hyphal structure (endohyphae) or from the cell wall and progressing externally (Fig. 3.5).

The hyphomycete genus *Gliocladium* is characterised by densely penicillate conidiophores which bear one-celled hyaline, smooth walled conidia in heads or in columns (Biourge, 1923; Raper and Thom, 1949). When found in columns the conidia have reduced but distinct connectives (Pitt, 1979). Some members of the *Gliocladia* have received attention as biological control agents for a variety of diseases (Lynch *et al*., 1987; Burpee and Martin, 1992; Yong *et al*., 1992). An important component of their ability to suppress certain soil pathogens is due to their rhizosphere competency and soil persistence (Howell and Stipanovic, 1995).

Within the genus *Gliocladium* several species have been associated with diseases of a range of crops: broad bean (Al-Hamdany and Salih, 1986); cashew (Esuruoso *et al*., 1974); cherimoya (Siddaramaiah *et al*., 1981); clover (Nan *et al*., 1991); and coconut (Mishra *et al*., 1989). Most notable in the genera in terms of its ability to induce disease, is *G. vermoesenii* (Biourge) Thom, a virulent pathogen of a variety of palm species (Marziano *et al*., 1980; Feather *et al*.,1979; Atilano *et al*., 1981) in many parts of the world. *G. vermoesenii* was observed to be very similar in morphology to the *N. psidii* isolates, as well as producing endogenous hyphae (Urbasch, 1984). *G. vermoesenii* Type 1 conidia were of a similar dimension (Table 3.3), but the Type 2

conidia were generally smaller. *G. vermoesenii* typically has a temperature optimum for mycelial growth of 25^0C, but is severely suppressed at 30^0C and is totally inhibited at 33^0C and 7^0C. High temperature inhibition of *G. vermoesenii* has been postulated as being a primary factor for the summer decline of pink rot disease of *Chamaedorea* palms in Florida (Atilano *et al*., 1980). Both the South African and Taiwanese *N. psidii* isolates grew well at 33^0C and maintained growth (albeit at lower rates) at temperatures of 38-39^0C (Chapter 4, Figs. 4.3 and 4.4).

G. vermoesenii exhibited only a slight growth response to light exposure (Table 3.4).

Table 3.4. Morphological and Physiological Comparison of *Gliocladium vermoesenii* and the South African Guava Wilt Fungus, *Nalanthamala psidii*

	G. vermoesenii	GWD pathogen Isolate CSF 221 *N. psidii*
Asexual spore types	2	2
Chlamydospores	6-10 μm	8-15 μm
Phialides		
Type 1	10-18 x 2-3 μm	15-35 x 2.5-4.5 μm
Type 2	17-30 x 2-3 μm	20-45 x 2.5-4.0 μm
Optimal growth temperature	25-26°C	30°C
Cardinal temperatures	Min 8°C	Min 8°C
	Max 38°C	Max 44°C
Light sensitivity.	SLIGHT	YES
Endogenous hyphae.	YES	YES
Hyphal knots.	YES	NO
Pathogenic on guava.	NO	YES
Mycelial color[a]		
PDA solid media	Pink	Pink
Submerged Czapek's	Green	Off white/beige

[a] Cultures were grown in 12 hour lighting cycles at a constant 30°C for ten days.

When grown in darkness the *N. psidii* isolates are largely unpigmented, and in this regard they are similar to *Acremonium diospyri*, the mycelia of which remain unpigmented when grown in darkness (Seviour and Codner, 1973). Sporulation of the *N. psidii* isolates were similarly affected by light duration and wavelength, as was *G. vermoesenii* (Chapter 4, Tables 4.1, 4.4 and 4.5). Morquer *et al.* (1963) postulated that *G. vermoesenii* is a transitional species due to its propensity to produce two conidial types, as well as differing in other morphological characteristics from other *Gliocladia*. The diversity of species in this genus has led to several species revisions of fungi originally assigned to this genus, for example *Gliocladium roseum* Bain, which is now classified as *Clonostachys rosea* (Link: Fr) Schroers (Schroers *et al.*, 1999). Domsch *et al.* (1980) noted that the genus was not well defined and was generally considered to be a highly polyphyletic genus. Rehner and Samuels (1994), reported that the genus contains distantly related fungi and shares this attribute with *Acremonium* (Glenn, *et al.*, 1996), which houses the persimmon wilt pathogen *Acremonium diospyri,* a pathogen closely related to *N. psidii*.

3.5.2 Genomic Taxonomic Studies

The fungal D2 DNA region of the 28S large sub unit has a highly conserved genome and as such, it is ideal for taxonomic identification of fungi to the species level (Evertsson, *et al.*, 2000). When these DNA sequence analyses are presented as neighbor joining phylograms, whereby overall relatedness is ranked vertically and sequence homology compared horizontally (Fig. 3.14), species separation is generally accepted when there is a 1% or greater sequence variance (Saitou and Nei, 1987; Studier and Keppler, 1988). Comparative analysis of

the parsimonious photograms generated in this study with those of Schroers *et al.* (2005) found that the data sets were very similar, and that there was a high degree of alignment in the taxonomic placement of the GWD pathogen (Fig. 3.15). The genomic sequence analysis of the GWD pathogen clearly supported the studies of Schroers *et al.* (2005). The outcome of the genomic studies is that the GWD pathogen can be confidently be reclassified as *Nalanthamala psidii*, which is closely related to the persimmon wilt pathogen, *N. diospyri*, and the palm pink rot pathogen, *N. vermoesenii* (Chapter 1, Table 1.1).

3.5.3 Pathogenicity Studies

The extensive colonization of the xylem vessels observed in the dead plant sections (Figs. 3.5, 3.6 and 3.7) was indicative of the systemic nature of this pathogen. In the aerial tissues of infected guavas, extensive tyloses were observed in advance of colonizing mycelia, whereas tyloses were generally absent in root tissue (Fig. 3.6). Amorphous plugging was observed in root tissue ahead of mycelia (Fig. 3.6) colonizing the xylem vessels. Both tyloses and amorphous plugging are implicated in plant defensive responses to limit the longitudinal invasion by a pathogen (Cohen *et al.*, 1983; Beckman, 1987). *Nalanthamala psidii* was observed to produce narrow hyphae that penetrated pits in the xylem walls (Figs. 3.5 and 3.6). These hyphae were observed in culture, often growing endogenously within degenerating, larger hypha. Interestingly, the pathogen moved in an upward direction further and more rapidly than in the downward direction, a feature common to many vascular wilting fungi (Bruehl, 1987) and an area of further research in later chapters of this thesis. The limited colonization of the wound site by *G. viride* was of interest in later investigations into the

protection of wound sites by biological control agents.

As discovered in Chapter 2, all the GWD and TGWD isolates of *N. psidii* were pathogenic to guavas (cv. Fan Retief), and they required wounding for successful infection (Table 3.2). Plant died within four months of inoculation, although many plants died sooner (Chapter 4, Figs. 4.5 and 4.6). None of the type reference cultures were pathogenic to guava (Table 3.2).

3.5.4 Genus Level Taxonomy

GWD in Southern Africa and Taiwan has been shown to be the same disease, caused by a hyphomycetous fungus. Preliminary ultrastructural studies indicated that the fungus is an ascomycete, based on the simple septal pore arrangement. However, to date no teleomorph has been associated with this fungus.

The combined morphological features of the fungus causing GWD are substantially different from the compared genera except *Gliocladium*. However, since the initial description of *Gliocladium* in the mid-19th century by Corda (1840), and its subsequent taxonomic treatment by Morquer *et al*., in 1963, this genus is still not sufficiently delimited, and a satisfactory species structure within it has not been determined yet (Domsch *et al*., 1980). As such, it was with some reservation that the GWD fungus was initially placed within the genus *Gliocladium*, even though it was the genus that at the time of this study most closely described it (Grech *et al.,* 1985). Therefore, whilst the fungus was initially ascribed to the genus *Gliocladium*, the presumption at that time was that it was likely that further research would necessitate revision

of the taxonomy of the GWD pathogen. This prediction has come to pass, with a combination of morphological and genomic data providing the definitive answers to this taxonomic question (Schroers et al., 2005).

Over the course of this study, this fungus had been assigned to several other genera by other plant pathologists. However, these have missed the key taxonomic features described above, and described in the recent revision of the GWD pathogen by Schroers et al. (2005), ascribing it to *Nalanthamala psidii*. Therefore these previous identifications can be dismissed as incorrect.

3.5.5   Species Level Taxonomy

The putative identification of the fungus causing GWD is measurably different from all reported species within the genus *Gliocladium*, and has remained so for more than 30 years. The fungus most closely resembled *G. vermoesenii* and had been identified previously as that species (Brady, pers. comm., 1983). However, none of the member species of the genus *Gliocladium* have been reported as being pathogenic on the plant genus *Psidium* or related Myrtaceae.

It was clear that fungus causing GWD was a new fungal species, and that it did not fit well within *Gliocladium*. Schroers *et al. (*2005), utilizing morphological and molecular techniques, reclassified the GWD pathogen as falling within the genus *Nalanthamala.* They also classified it as a new species, *N. psidii*.

In the studies of Schroers *et al.,.* (2005) chlamydospores were not observed, as they were in this studies (Fig. 3.13). In this study chlamydospores were observed in submerged cultures in growing media of high osmotic potential, as well as with ionic species that have a propensity to induce hyphal stress (ammonium). When *N. psidii* was grown in submerged cultures containing a medium of a lower ionic strength with lower ammonia levels, chlamydospores were rarely observed. This may account for the differences between this study and those of Schroers *et al.* (2005). The discovery that the GWD pathogen can produce chlamydospores is likely to be important for the long term survival of the fungus in soil.

In the DNA sequence studies conducted on *M. psidii*, *G. vermoesenii*, *A. diospyri* and *Rubrinectria* by Schroers *et al.* (2005) the authors proposed that these fungi should all be placed in the genus *Nalanthamala*. In the same studies, the authors also found differences in specific rDNA sequences from morphologically indistinguishable strains of the GWD pathogen from South African and Taiwan, possibly indicating separate lineages.

The findings reported in these studies support, and are generally congruent with, the findings of Schroers *et al.* (2005) in their revision of the GWD pathogen to *N. psidii*.

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CHAPTER 4: PHYSIOLOGICAL COMPARISON BETWEEN SOUTH AFRICAN AND TAIWANESE ISOLATES OF *NALANTHAMALA PSIDII* AND SPECIFIC COMPARISONS WITH *GLIOCLADIUM VERMOESENII*

ABSTRACT

South African and Taiwanese isolates of the guava wilt disease (GWD) pathogen *Nalanthamala psidii* (Biourge) Schroers have identical morphology and have taxonomic similarities to *Gliocladium vermoesenii* (Biourge) Thom, a fungus that previously had been identified as the GWD pathogen. Type culture isolates of *N. psidii* from South Africa (CSF 221) and Taiwan (CSF t226) grew similarly across a range of pH values, with cardinal values of <2.5 and >9.5, and an optimal pH range of 5.5 to 7.5. Both isolates have very similar growth characteristics across a range of temperatures. Cardinal temperatures for both isolates were 38-40°C (maximum) and 8-10°C (minimum). Lethal temperatures were 2°C and 46°C after a 15 minute exposure. *Gliocladium vermoesenii* had an optimum growth temperature of 25°C. The temperature optimum for conidial germination of *N. psidii* isolates showed a similar pattern to that obtained for mycelial growth. Type 1 and Type 2 conidia exhibited differences in their optimal germination temperatures, which were found to be 30°C and 27°C, respectively. Maximal germination at these temperatures was normally reached after 24 hours. All three fungi utilized in these tests were responsive in terms of sporulation to white and red light. Increasing relative humidity decreased the ratio of Type 1 to Type 2 conidia. Sucrose resulted in the maximum growth in culture of both *N. psidii* isolates as compared to any other carbon source tested. Both isolates grew well on organic nitrogen sources, especially asparagine. However, maximum mycelial growth was recorded on media containing potassium nitrate by both isolates. Overall, although some slight strain differences occurred in terms of temperature effects on growth and conidial germination, in terms of fungal physiology, the South African and Taiwanese *N. psidii* isolates are indistinguishable.

4.1 INTRODUCTION

Guava wilt disease (GWD) disease, caused by the fungus *Nalanthamala psidii* (Biourge) Schroers, is present in both South Africa (Grech, 1983; Schoeman *et al*., 2012) and Taiwan (Kurosawa, 1926; Hsieh *et al*., 1976; Leu and Kao, 1979; Grech ,1988), inflicting serious economic damage to the guava industries in these countries. The pathogen causing the disease in both countries appears to be taxonomically identical, and has been identified as *Nalanthamala psidii* (Schroers *et al*., 2005). However, there appear to be some strain differences between isolates. The aim of this study was to compare the physiological performance of the type cultures of *N. psidii* from Taiwan and South Africa, and also to compare them with the closely related fungus *Gliocladium vermoesenii* (Biourge) Thom.

4.2 MATERIAL AND METHODS

4.2.1 Physiological comparisons between the *N. psidii* causing GWD in Southern Africa and TGWD in Taiwan

Single spored isolates of the causal fungus of GWD and TWGD (CSF 221 and CSF t226) were collected by the author, as previously described above (Chapter 2), in South Africa and Taiwan. An isolate of *Gliocladium vermoesenii* (IMI 40321, sourced from CABI, Wallingford, UK) was included in the experiments investigating the effects of temperature and light on mycelial growth and sporulation.

These isolates were used to investigate the effect of various environmental conditions, as well as nutrition, on the growth and sporulation of these fungi. All tests were carried out in Czapek's broth or on agar media with a minimum of four replicates of the fungal isolate per treatment.

Each experiment was repeated at least twice. If the results between identical experiments were substantially different from one another, then the results from these are presented separately. Where results were consistent between experiments, then these data were averaged over the two experiments.

4.2.2 Hydrogen ion concentration (pH)

The South African and Taiwanese isolates of *N. psidii* (isolates CSF 221 and CSF t226) were compared in terms of their ability to grow in culture across a range of moderate pH values. Three buffers were utilized to stabilize pH drift: 2 g.L^{-1} citric acid used in the pH 3.5-5.5 range; 2 g.L^{-1} sodium dihydrogen phosphate used in the pH 6.0-7.5 pH range; and 2 g.L^{-1} TRIS (Merck) used in the pH 8.0-9.5 pH range. Corn meal agar (CMA) (Difco, Sparks, Nevada, USA) was prepared in 250 ml conical flasks, the buffers were added and the pH was adjusted from pH 3.0 to pH 9.5 in 0.5 increments by the addition of 2 M NaOH or 2M HCl. The media were autoclaved and plated into Petri dishes on a laminar flow bench. A spare flask of agar of each pH value was used to check that the autoclaving process did not affect the pre-sterilization medium pH. Both cultures of *N. psidii* were single conidial isolates. These were plated onto the CMA media by removing a 5 mm diameter plug of mycelium from 5 mm behind the advancing front of an expanding colony on PDA. Three replicates were used per isolate, per pH value. The cultures were incubated in darkness at 30$^{\circ C}$ and the final colony area was measured after 5 days. The pH of the spent media were also checked at the end of the experiment.

4.2.3 Temperature

Preliminary experiments were conducted to determine their approximate cardinal and lethal temperatures. Five replicate cultures of each isolate (CSF 221 and CSF t226) were grown on PDA in 9cm Petri dishes and placed in incubators in darkness at $10^{\circ C}$ and $40^{\circ C}$ respectively. Every 48 hours the cultures were assessed for growth and the temperature decreased by $2^{\circ C}$ (for the lower temperature regime incubator) and increased by $2^{\circ C}$ (for the higher temperature incubator). This process minimized temperature shock on the fungus because the incubators required up to 3 hours for temperature stabilization after the adjustment. The process was repeated until no further growth was recorded. At that point, sub-cultures were made and if the isolates were still alive, the temperature was modified as previously conducted, until no further evidence of culture viability could be obtained. On the basis of this experiment, ranges were determined to create temperature growth curves for both isolates.

Isolates were subjected to a temperature range of $10\text{-}42^{\circ C}$ in $1^{\circ C}$ increments. The cultures were incubated in the dark. The surface area of the isolates was measured daily for 7 days using a digitizing tablet (F-90, Apple Computers Inc., USA). At the end of the seven day incubation period, the Petri dishes were flooded with 10 ml sterile distilled water (SDW) plus a wetting agent (1% Tween[20]). The surface of the cultures was then lightly rubbed with a sterile camel hair brush to suspend the conidia. A sample of this suspension was then placed onto a haemocytometer and the mean ratio of Type 1 conidia (small, dry) to Type 2 conidia (long, wet) was calculated. This experiment was repeated except that the cultures were exposed to continuous illumination (40 watt incandescent light, Philips, Johannesburg), 30 cm above the

Petri dish cultures. Data on conidial ratios were collected from $12^{\circ C}$ to $42^{\circ C}$ in $3^{\circ C}$ intervals utilizing four Petri plate replicates per treatment.

4.2.4 The effect of temperature on conidial germination

The South African GWD isolate (CSF 221) and the Taiwanese isolate (CSF t226) were used to investigate conidial germination over the same temperature range as above (12-42$^{\circ C}$) in 3$^{\circ C}$ degree increments. Sporulating Petri plate cultures grown in darkness cultures in Petri dishes were rinsed under aseptic conditions with 15 ml SDW with 0.1% Tween[20] added and lightly rubbed with a sterilized camel hair brush. One milliliter of this conidial suspension was diluted in 99 ml SDW plus wetter and then 10 ml of this was spread onto WAA in Petri dishes and incubated at the prescribed temperatures. Germination was assessed at 24 hours after inoculating the Petri dishes. Two hundred conidia in total (Type 1 and Type 2) per replicate (4 replicate Petri plates per treatment) were counted microscopically by direct observation of the conidia on the agar surface. The mean number of germinated conidia of each conidial type was recorded and the conidial ratio determined.

4.2.5 The effect of incubation period on conidial germination

The South African *N. psidii* isolate (CSF 221) and the Taiwanese isolate (CSF t226) were used to investigate conidial germination on WAA at 30$^{\circ C}$ for various incubation periods (Table 4.3). Conidia were counted by direct observation of the agar surface utilizing a dissection microscopic at a magnification of 200-300X and assessed for germination. Four replicate Petri plates were used per treatment.

4.2.6 Effects of light on sporulation

CMA Petri dish cultures of a single spore isolate of *G. vermoesenii* as well as the GWD and TGWD isolates of *N. psidii* were grown at 30°C for seven days, under identical illumination conditions as earlier described, except that the diurnal duration of the lighting exposure periods were 0, 1, 2, 6, 12, 18 and 24 hours. Conidia were harvested, enumerated and germination was assessed on WAA at 24 hours after seeding the Petri plates.

Once the optimal lighting regime for sporulation of the GWD and TGWD isolates of *N. psidii* was determined by the previous experiment, isolates were grown under these conditions except that they were exposed continuously to 40 watt fluorescent bulbs (Philips, Eindhoven, The Netherlands) of different illumination colors [red, 680 nm; green, 540 nm; blue, 430 nm; ultraviolet 315-400 nm] for the same time periods as previously exposed. Petri dish cultures were placed 30 cm below the illumination source. Conidia were harvested from the entire Petri dish and counted utilizing a haemocytometer as previously described. Four replicate Petri plates were utilized per treatment.

4.2.7 Relative Humidity (RH)

Petri dish cultures (with the lids removed) of the GWD and TGWD isolates of *N. psidii* on CMA amended with 100 mg.L^{-1} penicillin were placed face down, in autoclaved desiccation jars (devoid of desiccants). The relative humidity (RH) in the desiccation jars was controlled by using a modification of the technique described by Johnson (1939), which entailed placing autoclaved solutions of glycerin at various solution strengths (and hence various specific gravities) in the jars. Fifty milliliters of the various RH solutions were used per chamber and were contained in a dish in the desiccation jars to provide a high surface area to volume ratio to achieve equilibrium in the internal RH of the desiccation jar quickly after the chamber was sealed by placing the lid back on the jar after lubricating the seals with petroleum jelly. Six RH levels were investigated (Table 4.7). Preliminary tests with a relative humidity meter (Spray Nozzle Pty, Ltd, Johannesburg) placed in the jars indicated that the RH was maintained within 5-10% of the specified levels over five days. The jars were incubated in darkness at 30$^{\circ C}$ and the final colony area being measured after 5 days. The abundance of the two types of conidia and their ratio to one another was assessed as above.

4.2.8   Utilization of different carbon and nitrogen sources and their effect on mycelial morphology and growth

Czapek's broth medium was prepared and dispensed into conical flasks (250 ml capacity) in 100ml quantities. The media was acidified to pH 6.0, stabilized by the addition of 0.68g potassium dihydrogen phosphate (0.1 M) as a buffer. The standard carbon or nitrogen source in Czapek's broth was substituted, as listed in Tables 4.8 and 4.9. All alternative carbon sources were made up to a concentration of 1% [(w/v) total equivalent carbon] in 100ml of medium. For the nitrogen sources the solution was made up to 0.1% [(w/v) total equivalent nitrogen]. Where inorganic sources were used to supply nitrogen, the base nutrient media composition was adjusted to account for the total mineral composition of the nitrogen supplying salt (e.g., potassium nitrate required that the potassium content of the base nutrient broth be adjusted accordingly to account for the potassium in the salt). The liquid growth medium was dispensed into conical flasks in 100 ml aliquots, each constituting a replicate.

Flasks containing the different treatments were inoculated with an 3 mm diameter plug of mycelium grown on 2% water agar of a cloned isolate taken from the edge of an expanding colony not more than 5 days old. The flasks were placed on an orbital shaker set at 30 r.p.m. and incubated at $30^{\circ C}$ for 22 days in darkness. At the end of the incubation period, mycelium was removed from the conical flasks, rinsed in SDW to remove excess broth and placed on pre-weighed double layered filtered paper. Excess water was drained from the mycelium by placing the loaded filter paper buckets (Whatman No. 1) over a Buchner funnel under partial vacuum. Dry weight measurements were determined after drying the mycelium and filter paper in a $50^{\circ C}$ oven for 24 hours. Two additional replicates were included in this study to examine

hyphal morphology as affected by these treatments. Four control sets were used in this experiment: inoculated Czapek's broth without a carbon source; inoculated Czapek's broth without a nitrogen source; inoculated complete Czapek's broth; and uninoculated complete Czapek's broth. Four replicates were used per treatment. Spent broth from all these experiments were refrigerated at

$1^{\circ C}$ for later use in toxicological studies.

4.3 DATA ANALYSES

Data were analyzed by one or two way analysis of variance and where appropriate, treatment means were separated using the Waller-Duncan *k*-ratio *t* test (Little and Hills 1990).

4.4 RESULTS

4.4.1 Hydrogen ion concentration (pH)

The pH of the growth medium had a significant effect on colony growth on CMA (Fig. 4.1). Cardinal pH values for *N. psidii*, were approximately 2.5-3 and 9.5-10 for both the South African and Taiwanese isolates. The optimal range was between 5.5 and 8.0, with maximal growth of both isolates occurring at a pH range of between 6.0 and 6.5 (Fig. 4.1). Mycelial morphology was affected by both high acidity and alkalinity. At pH values of 3.0-4.0 the mycelium was dense and thick walled, with a high incidence of endohyphae. Sporulation at this pH was much reduced. At high pH values the mycelia had less branching and was thinner walled than at low or neutral pH levels. The pH values of the spent media taken at the

termination of the experiment indicated little drift in the values, with a slight tendency to show a slight drop.
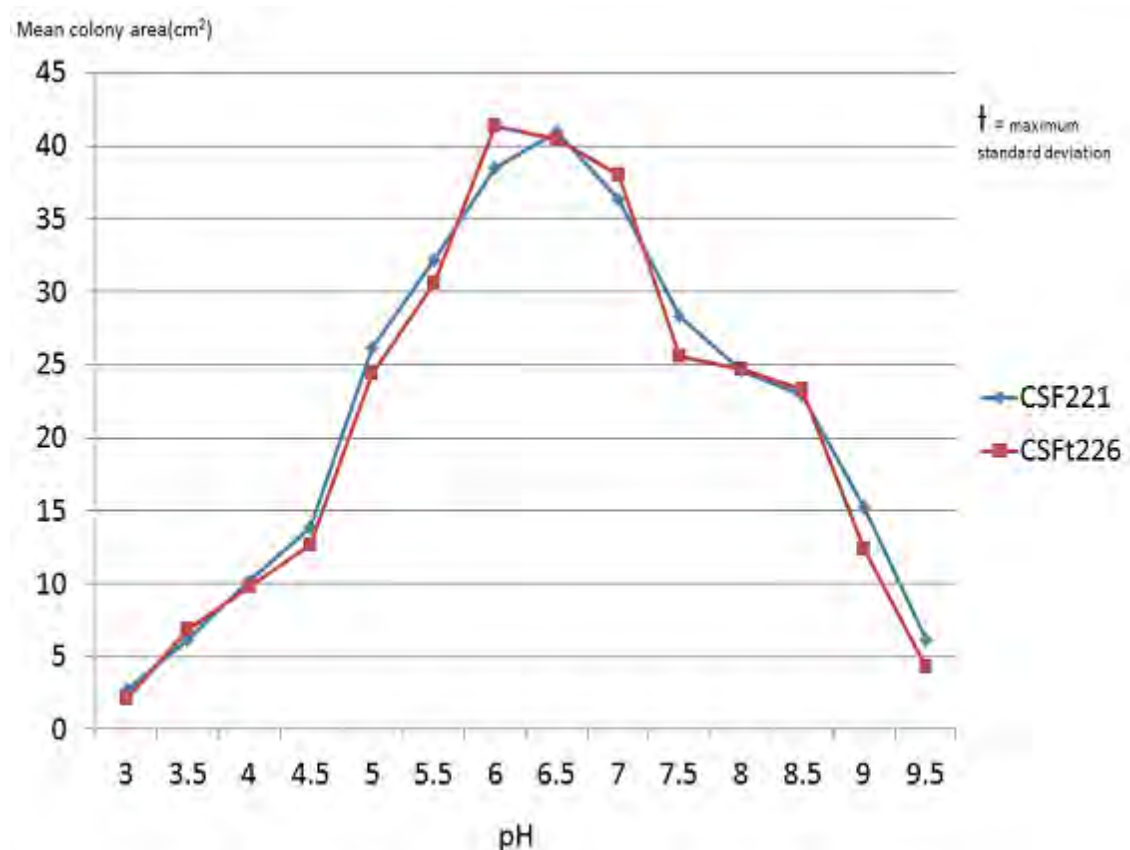
Figure 4.1. The effect of growth medium pH on the growth of the two type cultures of *Nalanthamala psidii*. Mean colony area after 8 days of growth on CMA at 30°C in darkness. Three replicates per treatment.

4.4.2 Temperature

The optimal temperatures for growth of the GWD and TGWD isolates of *N. psidii* were similar and both were approximately $30^{\circ C}$ (Fig. 4.2). In contrast, the tested strain of *G. vermoesenii* had a temperature optimum of $25^{\circ C}$ (Chapter 3, Table 3.3). The GWD and TGWD isolates of *N. psidii* stopped growing in the range of 8-$10^{\circ C}$. In the upper temperature range, their growth ceased at between $38^{\circ C}$ and $40^{\circ C}$ (Fig. 4.2).
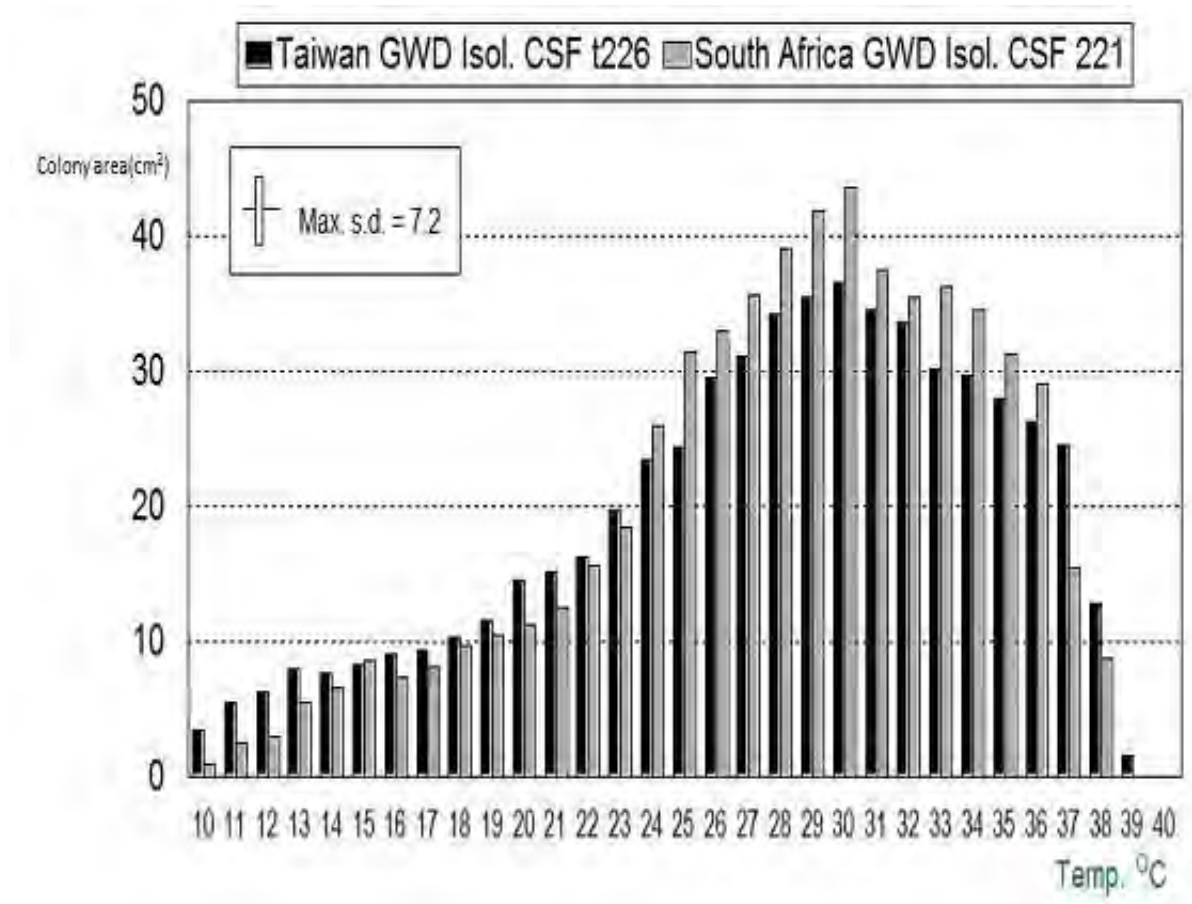
Figure 4.2. The influence of temperature on the growth of the South African and Taiwanese isolates of *Nalanthamala psidii* on PDA

Lethal temperatures for these isolates in agar or submerged cultures were $2^{\circ C}$ and $46^{\circ C}$ when they were exposed for 15 minutes or longer. Primary mean hyphal extension rates were 261 um.hr^{-1} at $30^{\circ C}$ on PDA. The ratio of the two types of conidia to one another was affected by temperature (Table 4.1), with the highest numeric ratio of Type 1 to Type 2 being recorded under illumination at $33^{\circ C}$ for both isolates. Light did not affect growth rates in culture, but did promote increased sporulation of the hydrophobic Type 1 conidia (Table 4.1).

105

Table 4.1. The effect of temperature and light on the numeric ratio of the Type 1 to Type 2 conidia of the isolates of *Nalanthamala psidii*, CSF 221(South African) and CSF t226 (Taiwanese)

Temperature T$^{°C}$	Mean Ratio of Conidial Type 1 to Type 2[a]			
	Isolate CSF 221		Isolate CSF t226	
	Light	Dark	Light	Dark
12	LG	LG	LG	LG
15	0.09a	0.03a	0.03a	0.05a
18	2.4b	0.3b	1.7b	0.2b
21	4.7b	0.2b	2.8b	4.4c
24	5.1b	1:2b	3.3bc	3.7c
27	9.8bc	8.5c	6.8c	5.5c
30	10.8c	9.4c	6.4c	5.8c
33	11.7c	7.5c	7.8c	5.8c
36	10.2c	7.1c	6.3c	4.7c
39	6.2b	4.8c	4.5bc	5.1c
42	1.6b	0.4b	1.2b	0.3b

[a] Four replicates per treatment, 200 conidia counted per replicate. Figures given are the numeric ratio of Type 1 conidia to Type 2 conidia. Illuminated cultures were exposed continuously. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). LG= Little measurable growth. The results are a mean of two experiments.

Temperature optima for conidial germination showed a similar pattern to that obtained for mycelial growth (Table 4.2). However, the two conidial types did exhibit marginal differences in their germination temperature optima [30$^{°C}$ and 27°C, respectively (Figs. 4.3, 4.4 and Table 4.2)]. The smaller hydrophobic Type 1 conidia had much higher germination levels after 24 hours compared to the Type 2 conidia (Table 4.2).

Table 4.2. The effect of temperature on the mean germination of Type 1 and Type 2 conidia, of the two type cultures of *Nalanthamala psidii*

Temperature	Conidial Type			
	Isolate CSF 221		Isolate CSF t226	
Temp (T°C)	Type 1	Type 2	Type 1	Type 2
12	LG	LG	LG	LG
15	14.1a	5.2b	0a	2.2a
18	45.3b	10.7c	18.0b	8.9b
21	62.6bc	13.3c	46.4c	7.4b
24	96.2cd	12.2c	88.6cd	10.2c
27	148.8d	13.0c	117.8d	12.1c
30	155.5d	12.8c	165.2e	10.0c
33	139.1d	7.1bc	153.9e	5.7b
36	110.4d	4.6b	135.4de	6.7b
39	73.8bc	1.9a	39.6bc	2.6a
42	11.4a	1.4a	0a	1.9a

[a] Four fungal culture replicates per temperature, 200 conidia counted per replicate. Figures given are the mean number of conidia that germinated per treatment. Germination was assessed at 24 hours after seeding the Petri plates. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). LG= Little germination. The results are the mean of two experiments.
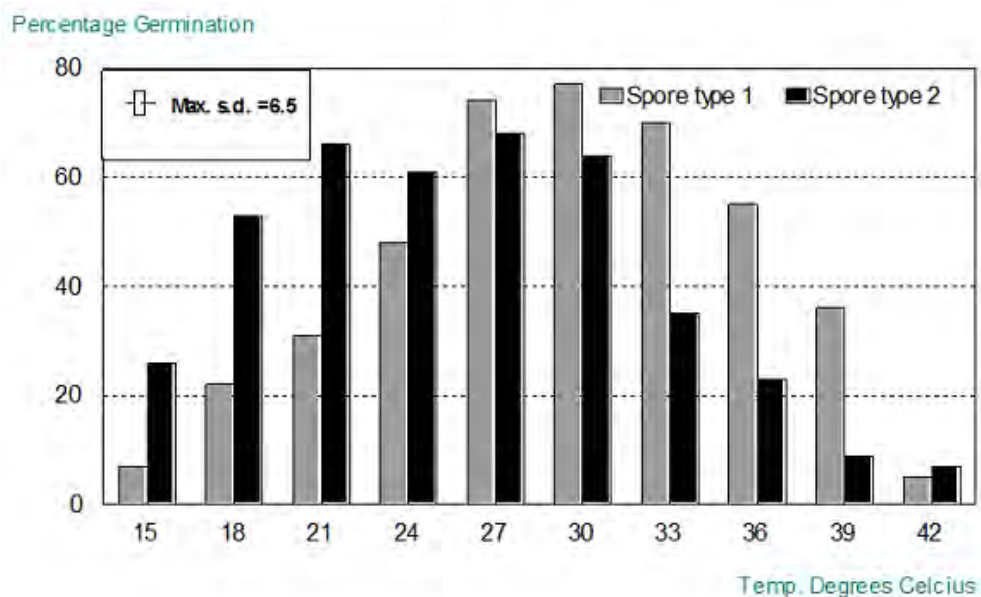
Figure 4.3. The effect of temperature on T1 and T2 spore germination of the South African isolate of *Nalanthamala psidii* (CSF 221) at 48 hours incubation.
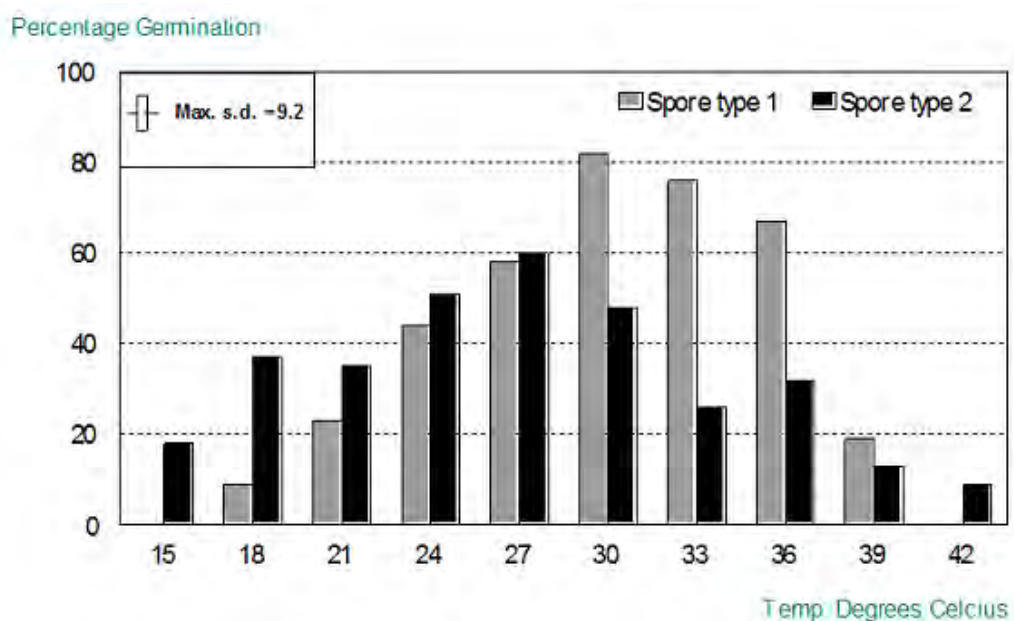
Figure 4.4. The effect of temperature on germination of T1 and T2 conidia of the Taiwanese isolate of *Nalanthamala psidii* (CSF t226) at 48 hours incubation.

4.4.3   Conidial Incubation Period

Germination rates of both conidial types incubated at 30°C reached their maxima at 24 hours incubation (Table 4.3). At the 48 hour assessment the percentage germination of Type 1 conidia was generally similar to the 24 hour assessment, but for the Type 2 conidia, germination was significantly higher (Table 4.3).

Table 4.3. The effect of the incubation period on the germination of the Type 1 and Type 2 conidia of the South Africa and Taiwanese isolates of *Nalanthamala psidii*

Conidial Incubation period (hours)[w]	Mean Conidial Germination[x]			
	CSF 221		CSF t226	
	T1	T2	T1	T2
0	0a	0a	0a	0a
2	23b	2b	17b	3a
6	48c	11bc	42c	17b
12	94d	14c	101d	21bc
18	107d	19c	126d	24bc
24	115d	27c	138d	32c
48	131d	67d	130d	71d

[w] Assessment of conidial germination at various incubation intervals post Petri plate seeding on WAA.
[x] Each figure represents the mean of four culture replicates; 200 conidia were assessed for germination per culture and the mean calculated. Conidia were incubated at 30°C. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). The results presented are the mean of two experiments.

4.4.4 Light

Light did not affect mycelial growth but did affect sporulation. *G. vermoesenii* exhibited similar

sensitivity to light as the *N. psidii* isolates. Type 1 conidial production was enhanced by

illumination (Table 4.1). Sporulation of the *N. psidii* isolates was directly proportional to the

duration of the light exposure up to 18 hours (Table 4.4), after which there was a trend towards

a reduction in total sporulation. The ratio of Type 1 conidia to Type 2 conidia was also greatest

at an incubation temperature of $33^{\circ C}$ under illumination (Table 4.1), reaching a maximum ratio

of 11.7 and 7.8 for the South African (CSF 221) and Taiwanese isolates (CSF t226),

respectively. Type 1 conidia dominated under all illumination regimes. In the absence of light,

Type 1 conidia were at statistically reduced levels from all other illumination regimes (Table

4.4).

Table 4.4. The effect of illumination duration on the sporulation in culture of the South African and Taiwanese isolates of *Nalanthamala psidii,* compared with that of *Gliocladium vermoeseniii*

Illumination period (hours)	Mean Total Sporulation^x (x 10^6)		
	CSF 221	CSF t226	*G.vermoesenii*
0	<5*a	<5*a	<5a
1	62b	55b	13b
2	98c	107c	35b
6	139cd	144c	77c
12	192d	187cd	86c
18	232d	254d	158d
24	225d	243d	172d

The figures indicate the mean total number of conidia per 9 cm petri plate per four culture replicates incubated at 30°C for 7 days.
* Very few Type 1 conidia observed.
Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Results presented are the mean of two experiments.

Conidia harvested from Petri plate cultures grown under different illumination periods did not

differ in their germination percentages (Table 4.5).

Table 4.5. The effect of diurnal illumination duration on conidial germination of the South African and Taiwanese isolates of *Nalanthamala psidii,* compared with that of *Gliocladium vermoesenii*

Culture Daily illumination period[w] (hours)	Mean Percentage Germinated Conidia[x]		
	CSF 221	CSF t226	*G. vermoesenii*
0	68a	60a	44a
1	62a	45a	53a
2	59a	58a	35a
6	76a	49a	77a
12	81a	77a	86a
18	79a	80a	88a
24	86a	79a	77a

[w] Duration of illumination of Petri plate cultures prior to germination assessment on WAA at 24 hours after seeding the Petri plates.
[x] The figures indicate the mean percentage of germinated conidia per 200 conidia counted per 9 cm Petri plate, replicated four times per illumination regime. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Results presented are the mean of two experiments.

The *N. psidii* isolates were insensitive to light wavelength in terms of the colony area on CMA. However, there was increased sporulation as a result of red spectral band illumination as compared to the other color spectral bands (Table 4.6). The fungi were insensitive to blue, green or longwave ultraviolet (black light).

Table 4.6. The effect of illumination of different wavelengths on the sporulation of *Gliocladium vermoesenii* and of the South African and Taiwanese *Nalanthamala psidii* isolates

Illumination type wavelength (nm)	Mean Total Sporulation $(\times 10^5)^x$		
	CSF 221	CSF t226	*G. vermoesenii*
Ultraviolet (315-400nm)	67a	43a	56a
Blue (430nm)	37a	55a	65a
Green (540nm)	77a	52a	44a
Red (680nm)	188b	167b	125b
White (400-700nm)	224b	196b	270c

[x] The figures indicate the mean total number of conidia per 9 cm Petri plate per four culture replicates. Cultures were illuminated for 18 hours daily. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).

4.4.5 Relative humidity

Total conidial count and colony area were unaffected by the different relative humidity levels. Both types of conidia were produced at all humidity levels; but the ratio of Type 1 conidia to Type 2 conidia increased with decreasing relative humidity (Table 4.7).

Table 4.7. The effect of relative humidity on the numeric ratio of Type 1 to Type 2 conidia, total spore count and the mean colony area of cultures of *Nalanthamala psidii* isolates.

Relative humidity (%).	Numeric ratio of spore types[x] Count ml^{-1} (x 10^5)		Total spore cm^2/5 days[y]	Colony area of CSF 221[z]
	CSF 221	CSF t226		
40	21.7a	22.8a	91a	29.3a
50	22.5a	26.0a	111a	31.1a
60	21.2a	24.6a	106a	29.7a
70	15.4b	16.7ab	98a	35.4a
90	11.8b	13.6a	155a	33.8a
100	9.1b	8.5a	141a	34.7a

[x] Figures donate the numeric ratio of Type 1 to Type 2 conidia. Each figure is the mean of four replicates; 200 conidia were counted per replicate and the numeric ratio of the spore types determined. [y] Total spore counts were determined by a haemocytometer. [z] Mean colony area of CSF 221 after 5 days. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).

4.4.6 Fungal Nutrition

Carbon and nitrogen utilization by the two isolates were similar in terms of recovered hyphal dry weights (Table 4.8 and 4.9). Both isolates exhibited maximum growth with sucrose as the carbon source. The growth response to organic nitrogen by the two isolates was generally better than with inorganic nitrogen, with the exception of potassium nitrate. The South African isolate (CSF 221), was observed to utilize potassium nitrate more efficiently than the Taiwanese isolate (CSF t226).

Table 4.8. The effect of different carbon sources on the dry weight of the South African and Taiwanese isolates of *Nalanthamala psidii* when grown in submerged culture

| Carbon source | Mean dry mycelia weight (mg)[x] | |
	CSF 221	CSF t226
Sucrose	94.6d	65.4c
Saccharose	81.3cd	52.2c
Glucose	44.3bc	38.9c
Raffinose	28.7b	35.8bc
Galactose	58.9c	54.7c
Sorbitol	57.2c	49.4c
Dextrin	65.4c	47.9c
Fructose	78.8cd	58.0c
Maltose	41.3bc	29.7bc
Rhamnose	36.0b	25.5b
Sorbose	29.5b	22.1b
Starch	34,5b	33.2b
Control	7.7a	4.9a

[x] Each figure is the mean of four replicate mycelial dry weight assessments. Isolates were grown for 5d at 30°C, in darkness. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Results presented are the mean of two experiments.

Table 4.9. The effect of different nitrogen sources on the dry weight of the South African and Taiwanese isolates of *Nalanthamala psidii* when grown in submerged culture

Nitrogen source	Mean dry mycelia weight (mg)^x	
	CSF 221	CSF t226
Asparagine	90.5cd	82.7cd
Glutamate	134d	68.7c
Ammonium nitrate	26.3b	34.8b *
Ammonium sulfate	22.5b	34.6b
Ammonium chloride	27.7b	33.0b *
Potassium nitrate	145.6d	102d
Sodium nitrate	78.6c	67.9c
Urea	27.4b	28.3b
Control	5.7a	6.3a

^{x} Each figure is the mean of four replicate mycelial dry weight assessments. Isolates were grown for 5d at 30°C in darkness. Treatments followed by the same letter in the same column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). * Indicates that extensive mycelia malformation occurred in these treatments. Results presented are the mean of two experiments.

Hyphal morphology was not affected by the different carbon sources, and chlamydospores were observed in all treatments except the uninoculated control. Conidial production (Type 1) was evident in all of the carbon source treatments as well as in the complete inoculated Czapek's broth control. Conidial production emanated primarily from mycelia adhering to the inner glass surface above the media. These effects were not evident in the uninoculated Czapek's broth controls, and were absent in or greatly reduced in the incomplete inoculated Czapek's broth controls.

Hyphal morphology was only observed to be distorted (thicker walled, more abundant septa and clavate) in the ammonium and urea based nitrogen sources, with the exception of ammonium sulfate (Table 4.9). Chlamydospores developed in all ammonium and urea containing treatments. Type 2 conidia developed especially in the potassium nitrate and

116

ammonium sulfate treatments. These effects were not evident in the inoculated complete Czapek's broth controls, the uninoculated Czapek's broth control or the incomplete inoculated Czapek's broth control.

4.5 DISCUSSION

Physiologically the two isolates of *N. psidii* reacted in a similar manner to various environmental conditions they were subjected to. The results were in agreement with the fungal physiological studies of Morquer *et al*. (1963) and Hsieh *et al*. (1976). The effect of pH on the growth of the two isolates of *N. psidii* on CMA, in terms of their mean Petri plate colony areas, were similar across a broad range of pH values (Fig. 4.1). At the low and high pH values, colony growth was significantly negatively impacted, an effect that is common within the fungi (Shrandt *et al.,* 1994). The pH of the media impacted gross cultural and ultrastructural morphology. At pH values below 4.0, thick walled mycelia and a reduction of aerial sporulation was observed. At high pH values, the mycelial density was lower than at pH 6.0-7.0, and considerably less branching was evident. These effects of pH on fungal mycelia have been reported from *Acremonium diospyri*, a fungus closely related to *N. psidii,* and a wilt pathogen of the American persimmon (Crandall and Baker, 1950; Wilson, 1963; McRitchie, 1979; Read and Seviour, 1984). Generally in submerged culture, the isolates of *N. psidii* caused a slight reduction in the pH of the media over the course of the incubation period, which is consistent with the findings of Dimond and Peltier (1945) for *Penicillium*. Previously (Chapter 3) as well as in earlier reports (Leu and Kao, 1979), *N. psidii* is reported as being xylem limited in the early infective stages of *N. psidii* and as such has to withstand typical acidic pH values of 4.5-6 associated with these plant tissues (Rengel, 2002). Both isolates of *N. psidii* were found to be

able to grow at these typical xylem pH values and as such would indicate a specific adaptation to colonization of these tissues.

Sporulation followed the same overall trend as mycelial growth in response to temperature (Figs. 4.2, 4.3 and 4.4). Germination of Type 1 conidia at $30^{\circ C}$ of both *N. psidii* isolates, numerically peaked between 24 and 48 hours (Table 4.3). At the 48 hour assessment, Type 2 conidial germination was maximal and interestingly continued for up to four days post seeding. Quantitative germination assessment beyond the 24 hour post Petri plate seedling interval proved to be problematic as extensive development of mycelia was observed on the agar surface and this negated accurate individual germling delineation. This may account for the anomaly in Table 4.3 where the 48 hour conidial germination assessment of the Type 1, CSF t226 isolate resulted in a reduced mean conidial germination. The assumption is that this was an enumeration error. In future assessment, a lower conidial loading of the Petri plate may mitigate this.

Germination of the Type 1 and Type 2 conidial types occurred optimally at two different temperatures [$30^{\circ C}$ and $27^{\circ C}$, respectively (Figs. 4.3, 4.4 and Table 4.2)] and this feature may be important in the different stages of the infection cycle, and the epidemiology of the pathogen. Type 2 conidia are commonly found associated with the rhizosphere (Chapter 3) where the temperature is normally lower than ambient (Edmunds, 1964). Type 1 conidia are normally produced on aerial parts of the plant (Grech, 1983, 1985a, 1985b), exclusively in sporodochia (Chapter 3, Fig. 3.16). Mean summer daily temperatures in the shaded canopy (sporodochia are usually found growing under diseased bark in the lower canopy) were

118

generally between 26-30°C, with high temperatures usually not exceeding 33-35°C (Chapter 5, Fig. 5.8). Under-canopy soil temperatures in the summer months were found to be relatively constant and were generally in the range of 20-22°C (Chapter 3). These temperatures correlate well with the optimal germination temperature ranges for both the Type 1 and Type 2 conidial types (Table 4.2). The ability of this fungus to produce two distinct types of conidia (as well as hyphae) in response to the environmental conditions associated with subterranean or aerial guava tissue colonization reflects a distinct adaptation of this fungus to its habitat. As reported in Chapter 2, *N. psidii* trends towards greater virulence at higher incubation temperatures (Chapter 2, Table 2.2).

The presence of illumination, the duration and the wavelength of the incident light during incubation affected sporulation. Light and red light caused an increase in total spore counts of both isolates of *N. psidii* as well as with *G. vermoesenii* (Tables 4.1, 4.4 and 4.5), suggesting that these fungi are photoreceptive in the 680 nm wavelength range. Idnurm (2013) reported on the ability of red light to impact signal transduction in *Aspergillus fumigatus* and modify genetic regulation.

Spore count increases due to illumination, were predominantly due to increases in Type 1 conidia. Colony pigmentation under illumination was salmon pink versus white when grown in darkness (Chapter 3). The closely related fungus, *N. diospyri,* has also been shown in culture to develop significant pigmentation when exposed to light (Seviour and Codner, 1973). Conversely, Type 2 conidia were favored by darkness during incubation, Germination of conidia derived from Petri plate cultures incubated under various illumination wavelength

bands, was unaffected.

Relative humidity had no effect on colony area growth. However, decreasing relative humidity increased the ratio of hydrophobic (dry), Type 1 conidia to Type 2 conidia (Table 4.7). This phenomenon may have a role in the etiology of this disease. Type 1 conidia are produced on the aerial parts of the plant on dying limbs that dry out (Hsieh *et al*., 1976; Grech, 1983). On the palm *Chamaedorea elegans*, pink rot development, caused by *G. vermoesenii,* has been shown to be inversely proportional to relative humidity (Feather *et al*., 1979; Marziano *et al*., 1980; Atilano *et al*., 1981; Feather, 1982).

Both *N. psidii* isolates responded in a comparable manner (Table 4.8 and 4.9) when grown in the presence of different carbon and nitrogen sources. Mycelial growth was maximal on sucrose, fructose and saccharose (as carbon sources). As for organic nitrogen sources, glutamate and asparagine were utilized equally well by both isolates (Table 4.9), producing the most biomass as compared to the inorganic sources, with the exception of potassium nitrate, which produced the greatest mycelial dry weights. These findings correlated well with earlier work on other species of *Gliocladium* (Morquer *et al*., 1963). Interestingly, the South African isolate of *N. psidii* produced approximately 40% more biomass than the Taiwanese isolate when grown on potassium nitrate. These findings are broadly in agreement with those of Hsieh *et al*. (1976), who found that the Taiwanese isolates of *N. psidii* grew more rapidly on nitrate nitrogen.

Similar aberrations in hyphal morphology were observed with both N. *psidii* isolates when

grown with ammonium salts or urea as the nitrogen sources. These aberrations may be the result of the toxic nature of ammonium to certain fungi as reported by Chang and Chung (1988). Unpublished research (Grech, 1984) has shown that this fungus can tolerate growing in liquid media of up to 12-15% (w/v) sucrose solutions, emphasizing its tolerance of environments of high osmotic pressure (such as in xylem vessels) in accordance with previous findings (Hsieh *et al.*, 1976).

These studies showed that the South African and Taiwanese *N. psidii* isolates are generally indistinguishable by physiological behavior, and clearly support their common taxonomic identity. Some slight strain differences occurred in terms of temperature effects on growth and conidial germination, but overall the trends were closely aligned (Table 4.2 and Figs. 4.2, 4.3 and 4.4).

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CHAPTER 5: A CHRONOLOGY OF THE SPREAD OF GUAVA WILT DISEASE IN THE REPUBLIC OF CHINA, TAIWAN, SOUTH AFRICA AND THE KINGDOM OF SWAZILAND: MULTISITE ORCHARD DISEASE PROGRESS AND ORCHARD SPORE TRAPPING

ABSTRACT

Guava wilt disease (GWD), caused by the fungus *Nalanthamala psidii* (Biourge) Schroers, was first identified in Taiwan in 1926, and has since been found in the Philippines, Thailand, Indonesia, Malaysia and putatively in India. To date, the disease has not been found in the Americas. In Southern Africa, GWD first appeared in the early 1980's and over a decade, it has reduced the area planted to guava in the Provinces of Mpumalanga and Limpopo by over 40%. Several orchards were monitored in Southern Africa and Taiwan over several years. *N. psidii*, once introduced, usually at the orchard periphery, spread non-randomly, primarily by root to root contact. Root anastomosis between tree roots was found to be common in guava orchards over 8 years old, and it was in older clonal orchards that the pathogen was found to spread most rapidly. Summer tropical storms generally resulted in multiple new disease centers in an orchard. Tree mortality in summer generally exceeded 1.0 trees.ha^{-1}.day^{-1}, dropping to < 0.5 trees.ha^{-1}.day^{-1} in winter. These seasonal differences are well correlated with the optimal temperatures for the growth of *N. psidii*. Type 1 conidia exhibited a diurnal pattern of passive release in infected orchards, primarily from sporodochial structures on the above ground tissues of moribund or dead trees. Maximum Type 1 spore releases occurred usually during periods when the ambient temperature was highest and the relative humidity lowest, consistent with earlier *in vitro* studies on conidiogenesis and spore discharge. Rain events suppressed Type 1 spore discharges for several hours. Infective propagules were detected in irrigation water in infected orchards.

5.1 INTRODUCTION

Guava wilt disease (GWD) is a devastating systemic fungal wilting disease caused by the fungus definitively named *Nalanthamala psidii* (Biourge) Schroers as described by Schroers *et al. (*2005).

As with Panama disease of bananas, the universal and almost exclusive use of one guava cultivar in Southern Africa, "Fan Retief", has to a great extent led to the precarious position the guava industry finds itself in today (Su *et al*., 1986; Stover and Simmonds, 1987). Fan Retief is not only highly susceptible to GWD, but it is a multi-purpose cultivar that has major limitations in its performance for horticultural purposes (Du Preez and Welgemoed, 1985). As part of a breeding program to develop new guava cultivars, disease resistance must be a primary breeding objective (Ito and Nakasone, 1973; Grech and Rijkenberg, 1987; Negi and Rajan, 2007). Coupled to this, improved measures for disease management and containment need to be developed.

To date GWD has only been reported to occur in Asia and Africa (Chapter 1, Fig. 1.4). Over the last 30 years, Malaysia was the only country where GWD was detected but not confirmed (Schoeman, unpublished). The disease has also been reported from other countries in Asia (Athipunyakom and Luangsaard, 2008), and there are unconfirmed reports from India (Misra, pers. comm., 2007[1] and Gupta *et al.*, 2010). Globally the disease initially appeared in countries that had developed guava production systems based on asexually propagated (clonal) plant material (Hsieh, *et al.,* 1976; Grech, 1987). The Republic of South Africa and the Republic of China, Taiwan, are examples of countries with a sophisticated

guava industry exclusively based on clonal guava varieties, which usually originated by selection from seedling orchards.

The aims of this research were to study the epidemiology of this disease in various geographical locations in orchards under different cultural management systems and to garner an understanding of the mode of transmission between and within orchards.

5.2   MATERIALS AND METHODS

A chronological record was assimilated as to the progress of GWD into the guava producing areas of Southern Africa over the period 1981-1992. A similar chronological disease incidence map was prepared for Taiwan over the period 1926-1990. All experiments conducted in Taiwan were established by the author during visits to that country in 1987, 1994 and 2002. Disease progress maps in Taiwan and Southern Africa were prepared. Additionally the progress of GWD in the northern provinces of South Africa (Limpopo and Mpumalanga) in terms of the area affected by GWD was assessed over the period 1983-1992. Three distinct GWD censuses were conducted in 1983, 1985 and 1992. An assessment of the total healthy guava production area as well as the area afflicted with GWD was undertaken. The total diseased production area was enumerated by recording the total area of the affected orchard surveyed, as opposed to the total diseased tree area within an orchard. Eight orchards were primarily used in this study (Table 5.1).

Table 5.1. Characteristics of eight guava orchards affected with GWD

Orchard Location	Orchard characteristics					
	Age	Ctv[a]	Irrig[b]	Plts/ha	Pruning cycle/yr	Initial GWD %[c]
1.Malelane	27	FR	ME	400	1	0.3
2.Louw's Creek	22	FR	F	400	1	10
3.Louw's Creek	4	FR	F	600	0	3
4.Hazy-view	18	FR	US	400	1	11
5.Bron-dal	20	FR	OS	500	1	10
6.Taisir (Taiwan)	8	LP	D	800	4	17
7.Nelspruit	28	FR	F	400	1	25
8.Malkerns (Swazi-land)	12	SL	D	2000	0	19

[a] Cultivar; FR= Fan Retief, LP= Laia-Pa, SL= seedlings. [b] Irrigation delivery method; F=flood, US= under canopy sprinkler, OS= over canopy sprinkler, D= dryland, ME= micro emitter.
[c] Initial infection level at the first orchard inspection by the author (total number of trees infected or dead as a result of GWD expressed as a percentage of the total number of trees in the orchard).

Field GWD infection in experimental plots was confirmed by frequent routine tissue isolations of *N. psidii* as outlined in Chapter 3.

5.2.1   Epidemiology

Disease incidence was monitored monthly in Orchards 1, 2, 3, 4, 5 and 7 and periodically (3-4 times per year) in Orchards 6 and 8 (Table 5.1). Disease distribution and progress maps were developed that charted the progress of GWD distribution in these orchards over time. Orchards were mapped at approximately monthly intervals for between one and two years (depending on the site, in some instances, orchards were mapped for longer periods). The mean daily tree mortality rate was determined over the assessed period by monitoring the disease progress in each test orchard in 4 large (> 1 ha) replicated plots. In each orchard, a tree mortality value, averaged over the plots was calculated. Tests for non-random disease spread were applied to these orchards by using the methods of Madden *et al.* (1982). These tests were applied to twenty columns of at least 30 trees in each orchard on a monthly basis. These tests were applied at least for two seasons. In Orchards 2 and 5, humidity and air temperatures were collected using a 7 day mechanical recording thermohygrograph, placed 2 m above the orchard floor on a platform in the center of 4 trees. Soil temperatures were recorded automatically by a drum chart recorder and soil probe placed 100 mm below the soil at the canopy drip line and positioned at the north west side of the tree. All recording devices were protected from rain by mounting them under a perspex roof that was open on all sides.

5.2.2 Spore trapping

- Two methods of spore trapping were employed to determine the incidence of *N. psidii* conidia in a guava orchard where GWD was present (Orchard 2). The first method utilized a Burkhard volumetric spore trap (Burkhard, Rickmansworth, Herts, England), a device based on the earlier research of Hirst (1952) into spore dispersal. The device was placed in the center of four trees on the advancing front of the disease. The spore trap was placed under a perspex cover that was open on all sides but that protected the device from rain. The device was housed on a concrete platform surrounded by an anti-theft chain-link fence. The device was set to sample at a rate of 70 liters of air per minute and was placed out in the field for five consecutive days during each season of the year and where possible, during dry weather periods. The device was powered by a long duration deep cycle battery. The collection medium consisted of glycerin jelly amended with 200 mg.L^{-1} of rose Bengal, 20 mg.L^{-1} copper sulphate monohydrate and 5g.L^{-1} of citric acid (all from Merck Chemicals, Johannesburg, RSA) smeared onto a 20 mm wide cellophane strip mounted on a rotating drum. After 5 days of continuous operation in the field, the cellophane strip was unloaded and cut into lengths that corresponded to 24 hours of operation (approximately 50 mm). These strips were observed under the light microscope for the presence of conidia at magnification ranges of X 200 - 800. Conidial densities were assessed for each of the five days in three hour intervals. After the microscopic examination, each cellophane strip was cut transversely into 4 pieces of equal length (12.5 mm) representing 6 hour periods. These strips were

placed face down on acidified water agar amended with 3 mg.L^{-1} iprodione (Rovral$^®$, Bayer Crop Science, Isando, RSA), 250 mg.L^{-1} ampicillin [an antibiotic (Merck Chemicals, Halfwayhouse, Johannesburg)] and 15 mg.L^{-1} rose Bengal (WAA$_{rar}$), in Petri dishes (one per plate) and incubated in darkness at 36°C . After 2 days the cellophane strip was removed (as modified from the method of Holtmeyer *et al.*, 1981). The Petri dishes were incubated for between 24 and 48 hours with frequent microscopic examinations to detect fungal colonies and growth, as well as assessing the suitability for sub-culturing of those colonies. When sufficient germination of collected conidia had occurred to allow for sub culture, thirty colonies per dish were aseptically removed and each colony transferred onto acidified CMA amended with 15 mg.L^{-1} rose Bengal, 250 mg.L^{-1} ampicillin and 3 mg.L^{-1} iprodione ($_a$CMA$_{rar}$) and incubated at 36°C for seven days. After incubation, microscopic identification of the cultures was undertaken.

The second method of spore trapping involved exposing $_a$CMA$_{rar}$ in 7 cm diameter Petri dishes for one hour during periods of elevated (12h00 to 15h00) field atmospheric conidial densities, as determined by the previous spore trapping method. Prior to field deployment, the method was tested under conditions affording less variation, using an indoor warehouse. Sporulating cultures of *N. psidii* on PDA, as well as recently collected branches that had unruptured bark blisters (Chapter 3, Fig. 3.16) were used in these experiments. Bark blisters were carefully ruptured using a scalpel to expose the Type 1 conidial masses emanating from sporodochia. The guava branch substrate on which the sporodochia had formed was placed facing down at varying distances (0.5, 1.0, 2.0 and 3.0 m) above a cluster of open

Petri dishes containing $_a$CMA$_{rar}$. The cluster of open Petri dishes comprised of groups of four Petri dishes positioned perpendicularly across each cardinal point (Fig. 5.1), at varying distances (0.5, 1.0, 2.0, 4.0 and 8.0 m) from the central vertical axis, underneath the conidial source. Similarly, sporulating cultures of *N. psidii* grown on PDA were also positioned separately above the $_a$CMA$_{rar}$ collection dishes.
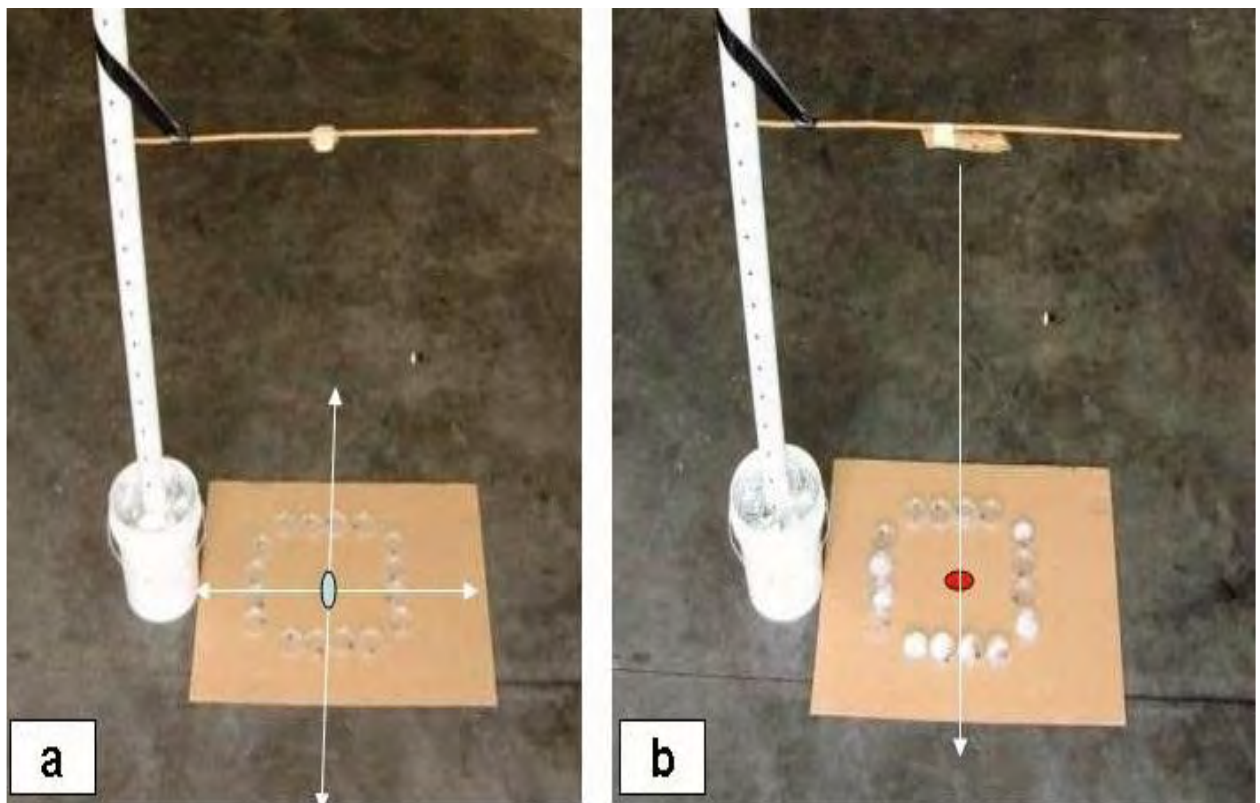
Figure 5.1. Apparatus to test the efficacy of passive conidial collection utilizing exposed media in petri dishes: a, apparatus loaded with a sporulating culture of *N. psidii*, the white arrows indicating the horizontal plane along which the petri dishes were positioned; b, apparatus loaded with a diseased guava branch that has exposed sporodochia, the white arrow indicates the vertical plane below which are the spore collection plates.

The exposed collection Petri dishes were left in position for 1 hour during the period 10h00

to 15h00. During this exposure period, the conidial sources were gently shaken every 15 minutes to simulate wind agitation of the guava canopy in the field. Initially the collection plates were positioned directly under the conidial source. The procedure was repeated at varying distances from the central axis as described above. After exposure, the Petri dishes were incubated at 36°C for approximately 24 hours and assessed for conidial germination and sub-cultured for taxonomic identification. In the field, Petri dishes of $_aCMA_{rar}$ were located at 0, 5, 10, 20 and 40 m along the cardinal points and centered in an orchard area of high disease severity. At each distance from the disease center, five Petri dishes were positioned at 2 m from one another perpendicularly across the compass cardinal points. This procedure was repeated on five consecutive days per season during dry summer periods in Orchard 2 over two years. Wind direction was recorded at each site. Collected Petri dishes were incubated at 36°C for two days and then processed identically to the first spore trapping experiment.

5.2.3   Spore content of irrigation water

An orchard that was flood irrigated was used in this trial (Orchard 2). The orchard was on a south-east facing slope and irrigation water from a canal at the top of the orchard was supplied to each tree row, from which it gravitated down to the lowest point. Typically the orchard was irrigated every two weeks in the dry season (winter) and monthly in the wet season (summer), unless a rainfall event occurred that negated the need for irrigation. During each season of the year, over two seasons, irrigation water was collected from the lowest point in a tree row furrow. Water was collected in this way from four tree rows. The water was collected in a surface sterilized bucket and poured through a series of wire mesh

strainers with a final mesh aperture of 50 μm. A total volume of 40 liters were collected from each of the four tree rows and placed in two 20 L plastic sealable pails which had been previously surface sterilized with a 0.5% sodium hypochlorite solution, after which they were rinsed with STW. Water was collected approximately 2 hours after the irrigation cycle commenced and processed within 2 hours. In the laboratory, the water samples were further filtered, over a partial vacuum, through a series of surface sterilized cartridge filters (Netafim, Kraaifontein, South Africa). The cartridge filters were first surface sterilized utilizing a solution of 0.05% NaOCl that was passed through them and after which they were rinsed with STW. The cartridge filters had pore apertures of 50, 20 and 10 μm. Finally, the 40 L water sample was split into four 10 L batches and each was filtered through a 40 mm diameter, 5 μm Millipore filter (Millipore Inc., Billerica, USA). The filter was removed, aseptically cut into 20 equally sized pieces and placed onto Petri dishes face down on $_a$CMA$_{rar}$ amended with 30 mg metalaxyl L^{-1} (for phycomycete suppression). Cultures were incubated for 48 hours at 36°C in darkness, after which the filter paper pieces were removed. The cultures were further processed in the same manner as for the volumetric spore trapping experiments.

5.3 DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, treatment means were separated using the Waller-Duncan k-ratio t test (Little and Hills 1990).

5.4   RESULTS

GWD was found in all the major production regions of South Africa, with the exception of the Cape regions (Fig. 5.2).
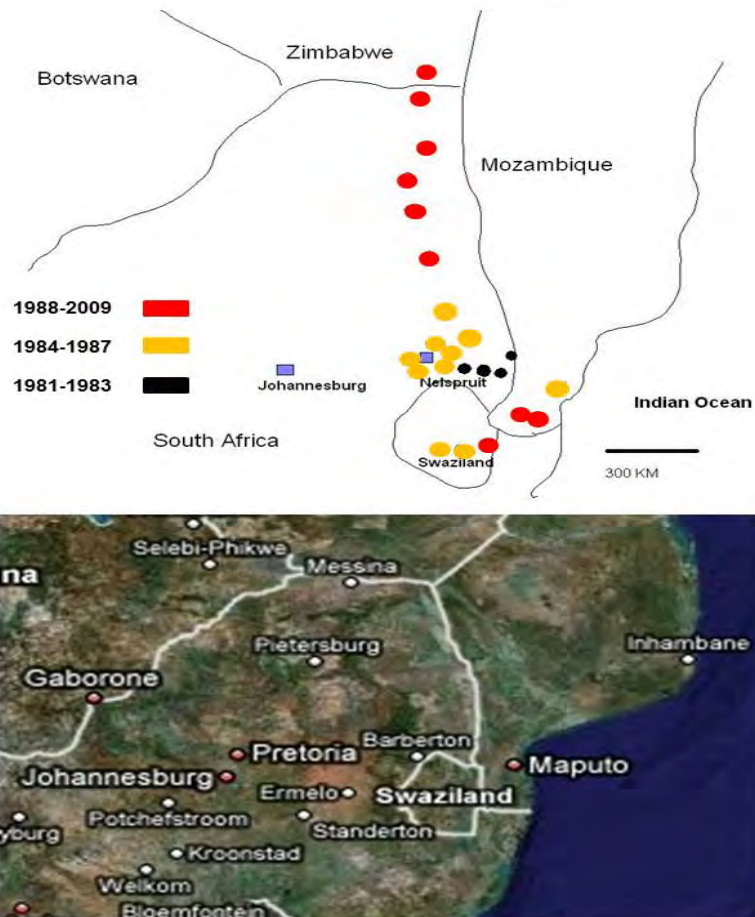
Figure 5.2. Distribution and chronology of reported cases of guava wilt disease in Southern Africa (1981-2009) encompassing South Africa, Zimbabwe, Swaziland and Mozambique.

Over a 10 year period GWD spread from the point of its first detection in Southern Africa (the Malelane/Komatipoort region of Mpumalanga) to Southern Zimbabwe

(approximately 500 km north), Maputo (150 km east) and Brondal (150 km west). The production area in South Africa affected by GWD over the period 1983-1992, increased in percentage terms from approximately 6% to 26% (Table 5.2) in these sub-tropical guava production regions. Guava cultivation area in the Mpumalanga and Limpopo regions of South Africa decreased by over 740 ha during the period 1983-1992 as a result of GWD. Currently the total guava production area in the Limpopo and Mpumalanga regions is approximately 600 ha.

Table 5.2. Surveys of areas affected by guava wilt disease in the Mpumalanga and Limpopo regions of South Africa: 1985-1992

Region	Total Area (Ha)[a]				Hectares Affected[b]		
	1983	1985	1992		1983	1985	1992
White River/							
Plaston	40	47	4		5	7	3
Brondal	125	154	6		6	19	10
Hazyview	71	82	11		0	20	0
Nelspruit	120	126	0		2	6	0
Komatipoort	20	27	0		3	5	0
Hectorspruit	3	3	0		0	0	0
Malelane							
Kaapmuiden/	93	120	0		10	45	0
Boulders	35	18	0		2	10	0
Richtershoek	49	49	0		0	0	0
Hoedspruit	30	35	0		0	15	0
Louw's Creek	40	43	0		15	28	0
Barberton	30	30	0		0	11	0
Tzaneen	306	345	277		0	0	55
Levubu	175	175	153		0	0	22
Total	1137	1254	451		43	166	90

[a] Total production area
[b] Area of cultivation within a region affected by GWD during the indicated period (assessed as the total area of afflicted, discrete orchards). This appears to be the first records for Zimbabwe and Swaziland.

In all of the eight orchards used in these experiments, *N. psidii* was routinely and

consistently isolated.

5.4.1 Epidemiology

On all the farms surveyed, the initial incidence of GWD was found at the orchard periphery,

usually bordering a road or an access point (Figs. 5.3-5.7 and Chapter 6, Fig. 6.2).

Figure 5.3. Aerial view of the spread of guava wilt disease. The position where the disease was first detected in the orchard is designated by the letter 'X'. This photograph depicts the disease progression, 14 months after the first diseased tree was identified. The orchard is located in the vicinity of Komatipoort close to the Mozambique border.
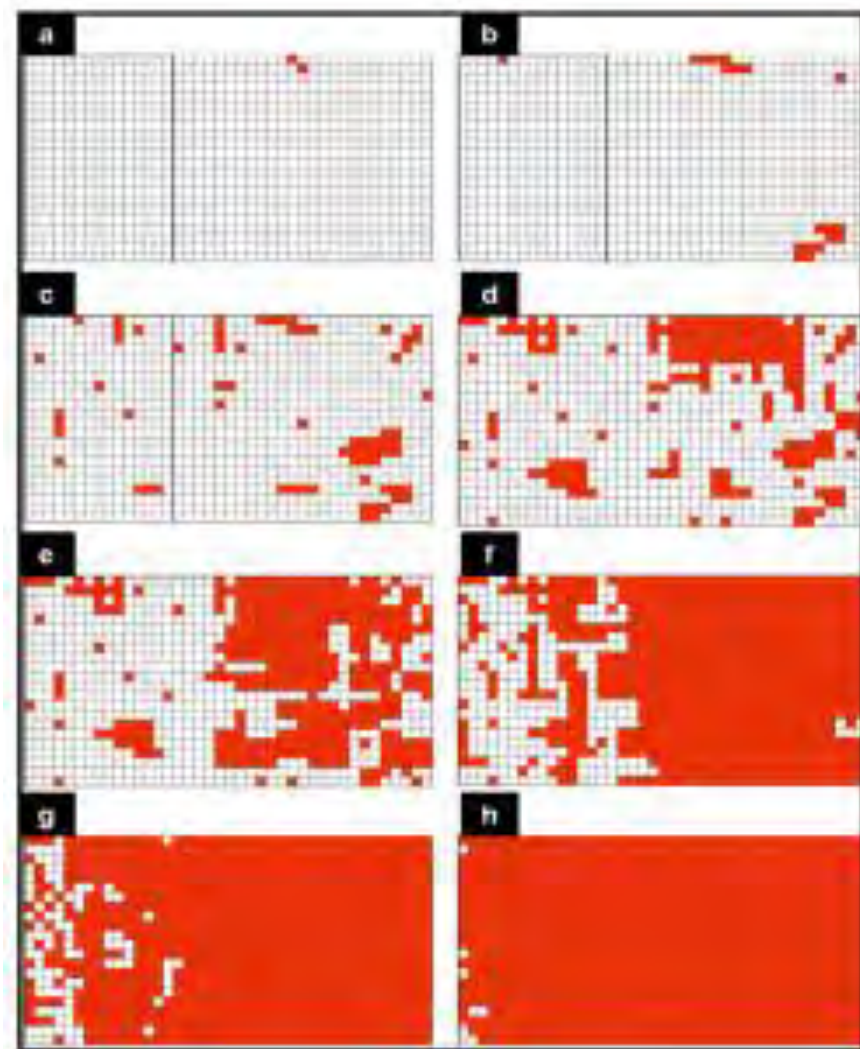
Figure 5.4. Monthly guava wilt disease progress map in Orchard 1 as recorded from the initial identification of two infected trees: a, January (T= month 1); b, April (T= month 4); c, July (T= month 7); d, October (T= month 10); e, January (T= month 13); f, April (T= month 16); g, July (T= month 19); h, October (T= month 22). Orchard area = 2.2 ha. Total trees = 880. Tree mortality by month 22 = 872.
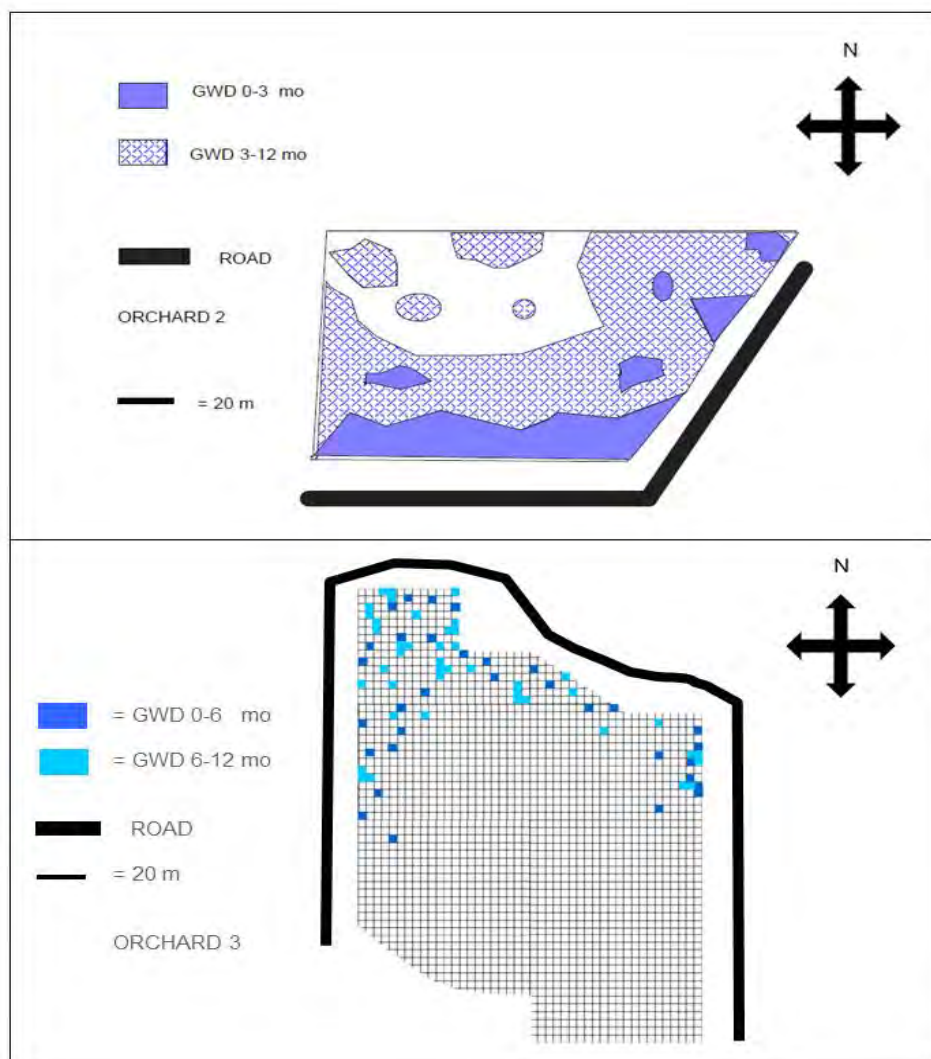
Figure 5.5. Chronological progress of guava wilt disease. Top = Orchard 2.
Bottom = Orchard 3.
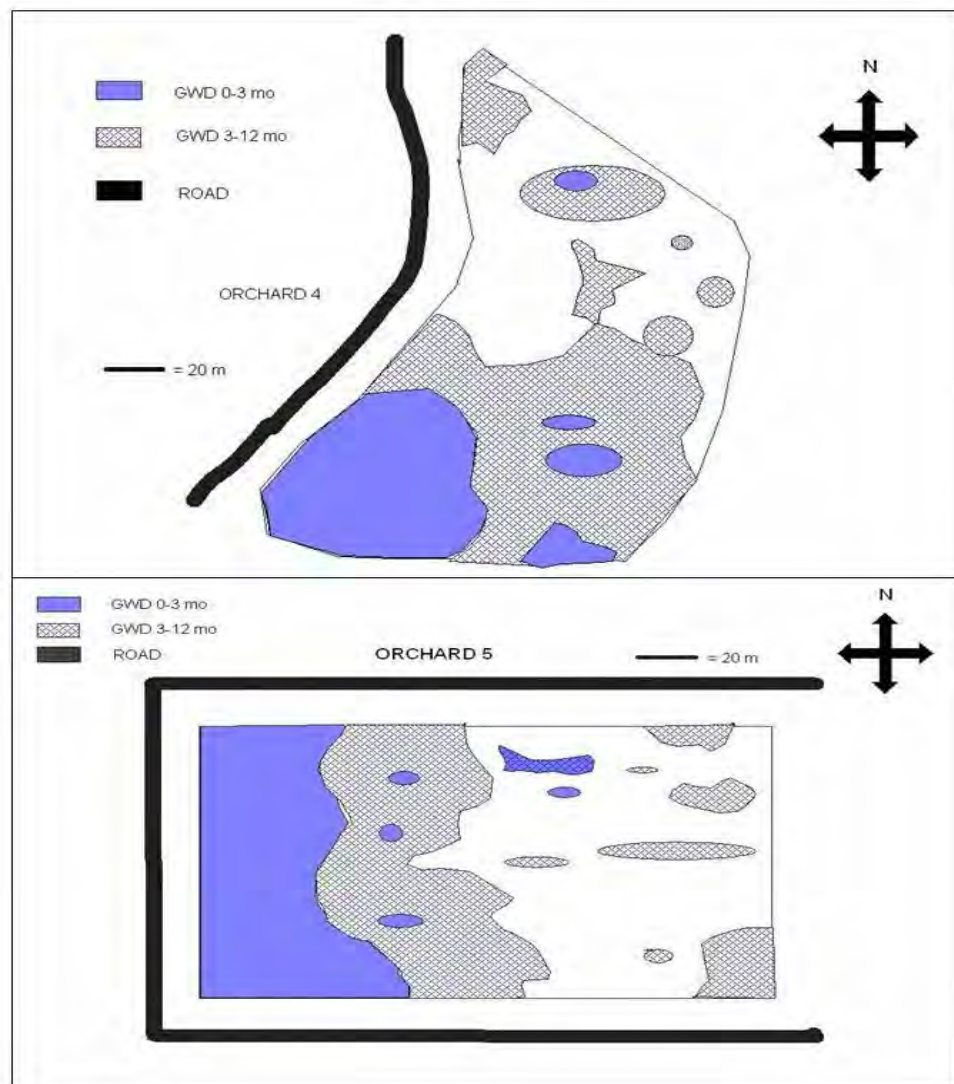
Figure 5.6. Chronological progress of guava wilting disease. Top = Orchard 4.
Bottom = Orchard 5.
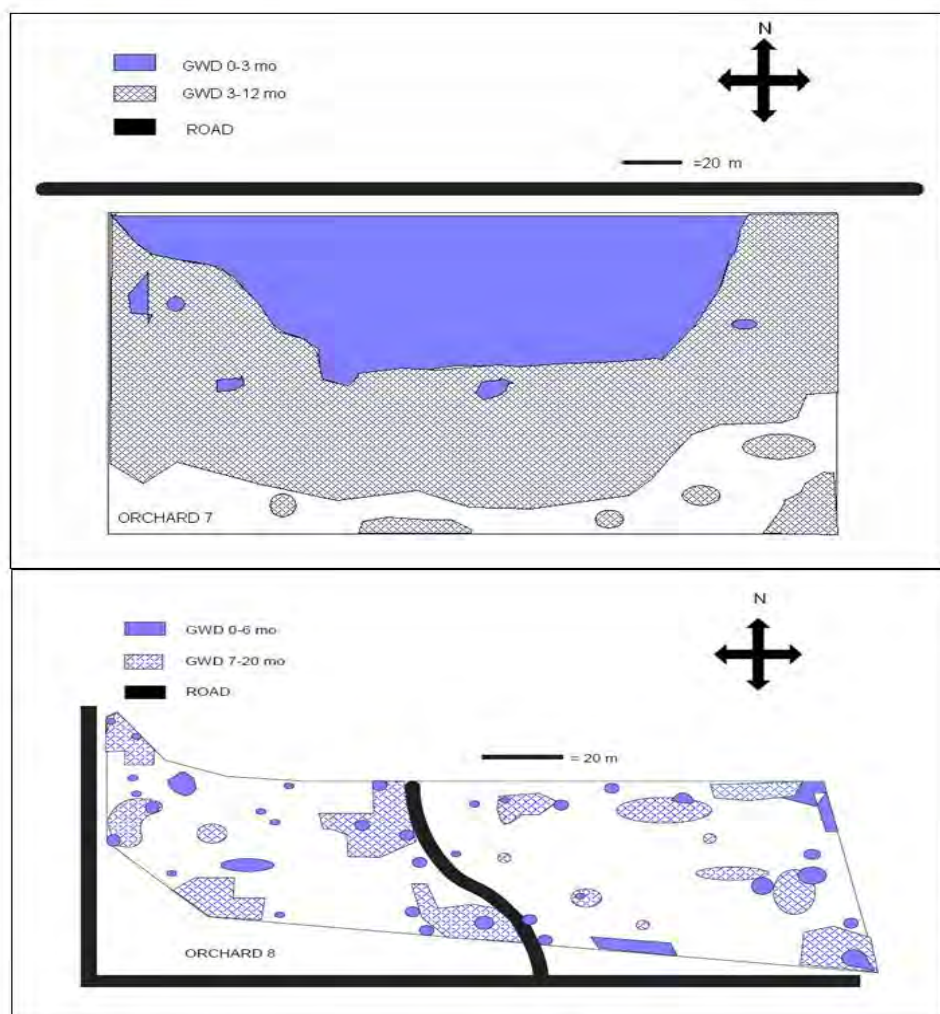
Figure 5.7. Chronological progress of guava wilt disease. Top = Orchard 7.
Bottom = Orchard 8.

From the initial disease locus, GWD generally spread rapidly along tree rows (Figs. 5.3-
5.7). Over the course of these studies, new disease loci appeared in the orchards, but the
major disease front moved predominantly from the initial disease centers, non-randomly
from tree to tree (Table 5.3). Monthly daily mortality rates indicated a distinct seasonality
in terms of tree death (Fig. 5.8).
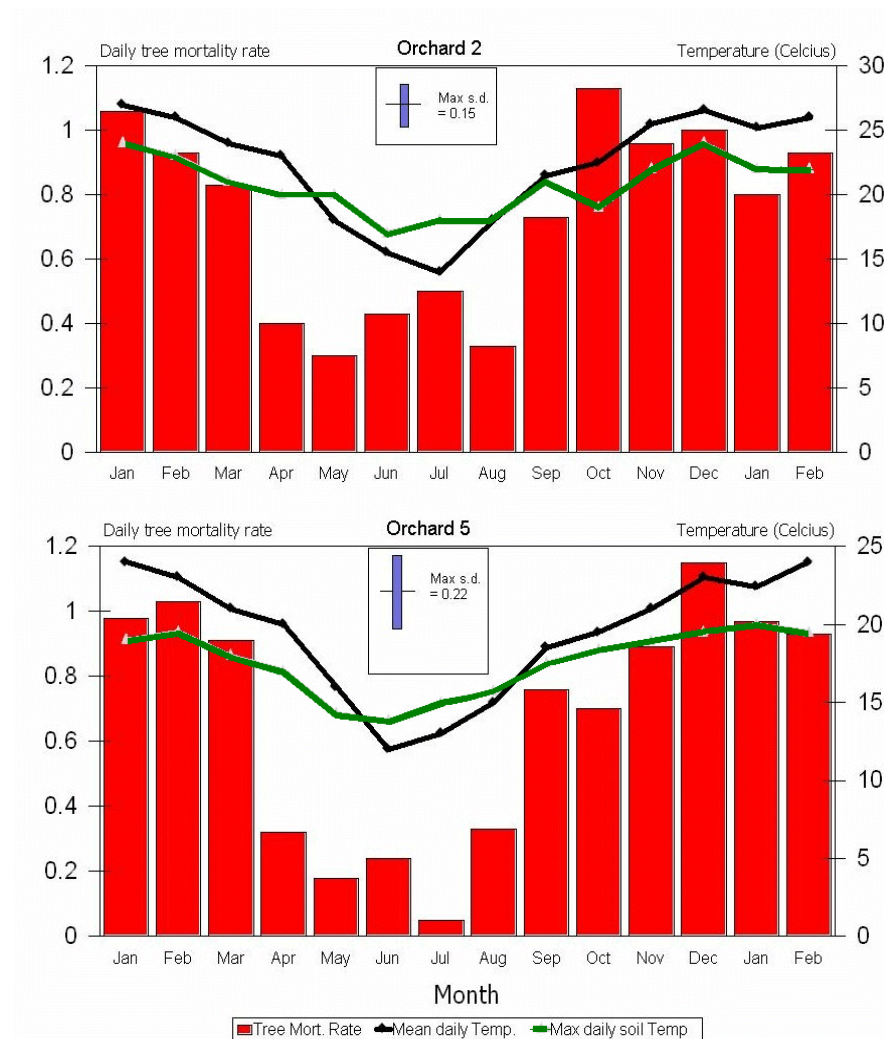
Figure 5.8. Mean daily air temperature, mean daily soil temperature and the mean daily tree mortality rates recorded monthly in two guava orchards infected with *Nalanthamala psidii* (top = Orchard 2, bottom = Orchard 5).

Daily tree mortality rates were lowest in Orchards 6 and 8 (Table 5.3), which were the only sites that were not planted with the extremely susceptible variety 'Fan Retief'; Orchard 6 was planted with the moderately susceptible variety Laia-pa. Orchard 8 was planted with open pollinated seedlings from the 'Fan Retief' selection.

Table 5.3. Tree mortality rates and statistical patterns of disease spread in eight GWD sites

Orchard number	Mean daily per ha tree mortality[a].	Test for random or non-random spread of GWD. (Runs analysis[b]) $-Z_u$	Result
1	1.6	- 160.4	NR
2	0.73	- 141.8	NR
3	0.56	- 42.7	NR
4	0.61	- 67.4	NR
5	0.68	- 91.6	NR
6*	0.36	- 134.2	NR
7	0.63	- 152.7	NR
8	0.31	- 24.7	NR

[a] These values represent the mean daily orchard mortality rate. The experiments were repeated twice and these data represent the mean values over two seasons. [b] As described by Madden *et al.*, (1982), the value, Z_u, is the sum of the Z-statistic equation. A column of plants is considered to have a non-random sequence of diseased and healthy plants if $-Z_u$ is greater than -1.64 (P=0.05). Each figure represents the mean value of 20 columns of at least 30 trees per orchard. NR = non random. * data only collected in this orchard for seven months.

5.4.2   Runs Analysis of disease spread

In all orchards surveyed, clustering of infected plants occurred, which could be measured

using Runs Analysis (Madden *et al.*, 1982):

(1) $E(U) = 1 + 2m(N-m) / N$

Where:

N = the total number of plants

m = the number of infected plants in a row or column

U = the number of clusters of either diseased or healthy plants

E = the number of expected clusters (or runs) if disease spread is random.

Under the null hypothesis of randomness (Madden *et al.*, 1982) the observed number of runs would be less than expected if clustering occurred in columns or rows of trees. The standard deviation of U is given by:

(2) $S_u = (2m(N-m)[2m(N-m)-n] / [N^2(N-1)])^{1/2}$

The standardized U is given by:

(3) $Z_u = [U+0.5-E(U)] / S_u$

According to this equation, a column of trees is considered to have a non-random distribution of healthy and diseased trees if $-Z_u$ is greater than 1.64 (P=0.05). As the Z-statistic equation (3) does not follow a normal distribution for N<20, hence all replicate columns in orchards were of at least 30 trees. In most cases GWD in an orchard moved along tree columns. Only in Orchard 2 were differences observed between the downward and upward movement of GWD in a tree row, which is probably related to the orchard

143

topography. Movement of GWD down the orchard gradient was much greater than in the upward direction.

Mean daily mortality rates per hectare were calculated for all orchards (Table 5.3). In Orchards 2 and 5 daily mortality rates during each month were calculated and graphically presented (Fig. 5.8). Rates of daily tree mortality assessed each month were highest in summer and lowest in winter, when mean daily temperatures were in the region of 14°C (Orchard 2) and 12°C (Orchard 5), as shown in Fig. 5.8.

Generally the tree mortality rate was lower in younger orchards (Table 5.3). The daily tree mortality rate averaged over two growing season was lowest in the non 'Fan Retief' Orchards 6 and 8 (Table 5.3). Unfortunately, in Orchard 6 seven months into the trial, the farmer decided to remove all dead trees and replace them with different varieties of guava and therefore no further data were collect from this orchard.

5.4.3 Aerial propagule recovery

The volumetric (active) spore trapping produced distinct diurnal patterns of fungal conidia (Fig. 5.9). Maximum levels of trapped fungal conidia usually occurred during 12h00 to 15h00 when the ambient relative humidity was generally at its lowest. Rain initially reduced the numbers of trapped conidia, but this was followed by an elevated recovery two days after rain (Fig. 5.9).

144
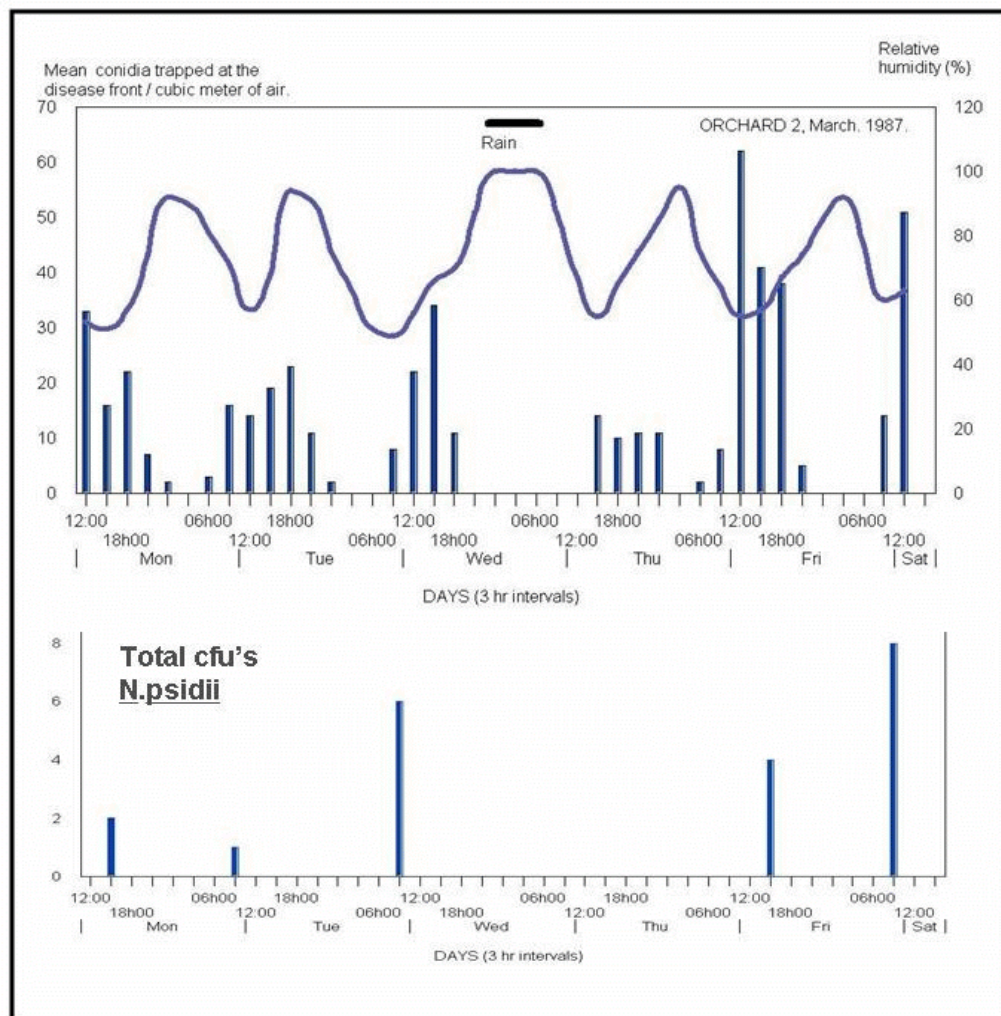
Figure
5.9. Top: daily variation in relative humidity and total trapped spore counts over a five day period in a GWD infected guava orchard (Orchard 2). Bottom: total colony forming units of *N. psidii* recovered.

Conidia of *N. psidii* could not be identified specifically by direct microscopic examination of the collection strips of the volumetric spore trap. A wide variety of easily recognizable fungal conidia were detected such as *Alternaria* spp. and *Fusarium* spp. Abundant *Penicillium*-like conidia were observed, many of which were morphologically and dimensionally similar to the *N. psidii* Type 1 conidia. Type 2-like conidia were not observed on the collection tape. In subsequent culturing of fungi from the collection strips, *N. psidii*

was isolated, albeit at low levels (Fig. 5.9) and most frequently during periods of low relative humidity, which followed the general pattern of conidial deposition on the collection tape of other aerially dispersed fungi, as mentioned previously.

Initial pre-field deployment tests for the passive collection of *N. psidii* conidia (Fig. 5.1) indicated efficient levels of conidial capture, the magnitude of which was numerically directly proportional to the proximity of the inoculum source and the collection dishes (Table 5.4).

Table 5.4. Mean N. *psidii* conidial recovery in pre field deployment passive
 spore collection efficacy tests utilizing exposed amended CMA in petri dishes

	Mean spore count on agar plates/m^2 [a]			
Horizontal distance of the collection plates from the central axis (m).	Vertical elevation above collection plates (m).			
	0.5	1	2	3
0.5	180 a	212 a	63 a	118 a
1	215 a	28 a	159 a	276 a
2	36 a	147 a	98 a	32 a
4	0 a	28 b	71 b	23 b
8	0 a	112 b	0 a	0 a

[a] Mean percentage spore count per m^2 of collection media. Values represent the mean recovery over 12 collection petri plates. Means followed by the same letter in each row do not differ significantly from one another (P=0.05), according to the Waller-Duncan *k*-ratio *t* test.
Numerous conidia were observed germinating on the collection media and upon transfer of the germlings, positive identification of the fungus was possible within 1-2 days.
In contrast to the pre-field deployment tests, the field Petri dish spore trapping method was not effective in trapping conidia of *N. psidii. Nalanthamala psidii* was not identified on any field exposed collection Petri dishes, directly or in subsequent sub-cultures.

During these studies it was observed that the prevalent winds were normally from the north-west, although during summer, tropical storms would move from an easterly direction. Wind did not seem to affect the spread of GWD (as determined from disease mapping) in the orchard except when summer tropical storms occurred. Several new disease loci would often be evident in the orchard several weeks after a storm due to extensive canopy damage.

5.4.4   Irrigation propagule recovery

Propagules of *N. psidii* were isolated from irrigation water at low levels (Fig. 5.10) and not during all seasons. These data indicated recoveries only in summer in both collection years. In the second year, the recovery of *N. psidii* propagules in summer irrigation water was much higher than in the first collection year. Three days prior to collection of the 2nd season December irrigation samples, the orchard received approximately 20 mm of rain. Orchard 2 was in a warmer production region and *N. psidii* was active in all seasons.
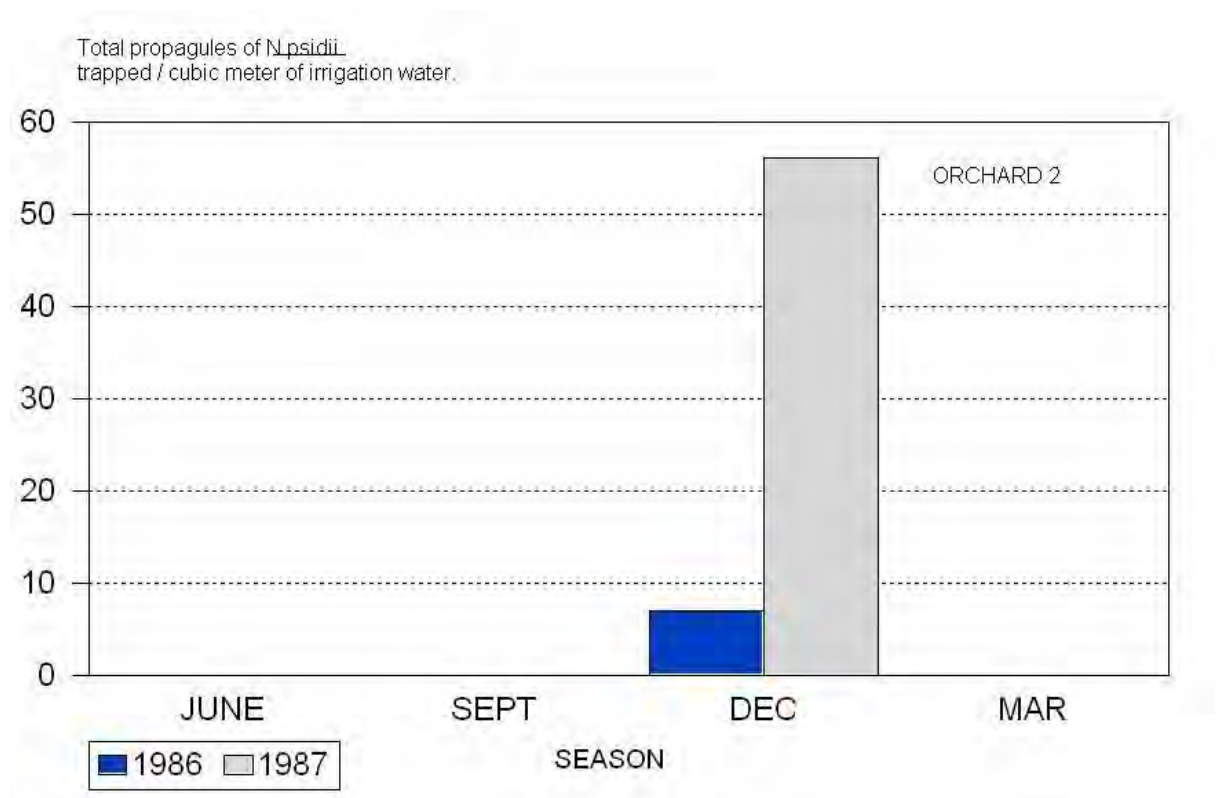
Figure 5.10. Seasonal incidence of *N. psidii* in irrigation water from an orchard infected with *Nalanthamala psidii* (Orchard 2). Data were collected over two growing seasons.

5.5   DISCUSSION

The area of guava production in the Mpumalanga and Limpopo provincial regions declined by over 40% over the period 1983-1992 as a result of GWD (Table 5.2). The situation specifically in the Mpumalanga Province over the same time period illustrates the greater impact of GWD in this guava region than the northerly production regions: +/- 750 ha in 1982 to 160 ha in 1994 (Anonymous, 2008). As of 2012, the total area under guava cultivation in both of these provinces is approximately 600 ha (Schoeman *et al*., 2012). During the three censuses, it became apparent that GWD outbreaks in a previously unaffected production region, rapidly resulted in the destruction of the entire production region within several years (Table 5.2). The rapidity of spread of GWD is clearly indicative of the highly contagious nature of this disease as well as the striking susceptibility of the 'Fan Retief' guava variety.

In many developing countries guava is viewed as a low input crop, consumed locally by a domestic market, and the lack of crop homogeneity is not seen as a serious impediment to guava production. Guavas are more easily propagated from seed than clonal propagation, methods, which require higher input costs and improved infrastructure. Hence in many developing countries, production is based on open pollinated, heterozygous seedlings (Morton, 1989; Negi and Rajan, 2007). As such, production and quality of fruit is variable. However, the lack of genetic uniformity has the beneficial effect of imparting a lower vulnerability to epidemics of GWD and perhaps this is a major contributing factor in the much slower geographical spread of the disease in Asia as compared to Southern Africa. In many parts of the tropics and sub tropics, plant material movement across borders is largely unregulated due to under-developed phytosanitary regulations, non-existent border

controls or both (Abdallah and Black, 1998). Unregulated movement of guava plant material is commonplace in many parts of Africa and Asia. As such, the likelihood that GWD has spread into previously unreported locations is a possibility. However, given the widespread use of guava seedlings in orchards in many parts of the world (Morton, 1989), broad genetic tolerance to GWD is also a possibility and one that is likely to retard the development of epidemics of the magnitude witnessed in South Africa and Taiwan. This argument is supported by the observation in these studies that the comparative tree mortality rates between test orchards showed that seedling-based orchards had the lowest annualized daily tree mortality levels (Table 5.3), even though these seedlings were derived from a susceptible parent ('Fan Retief').

GWD was first reported from the Republic of China, Taiwan in 1923 (Kurosawa, 1926) and over several decades it spread into all of the guava production regions of that country (Fig. 5.11).
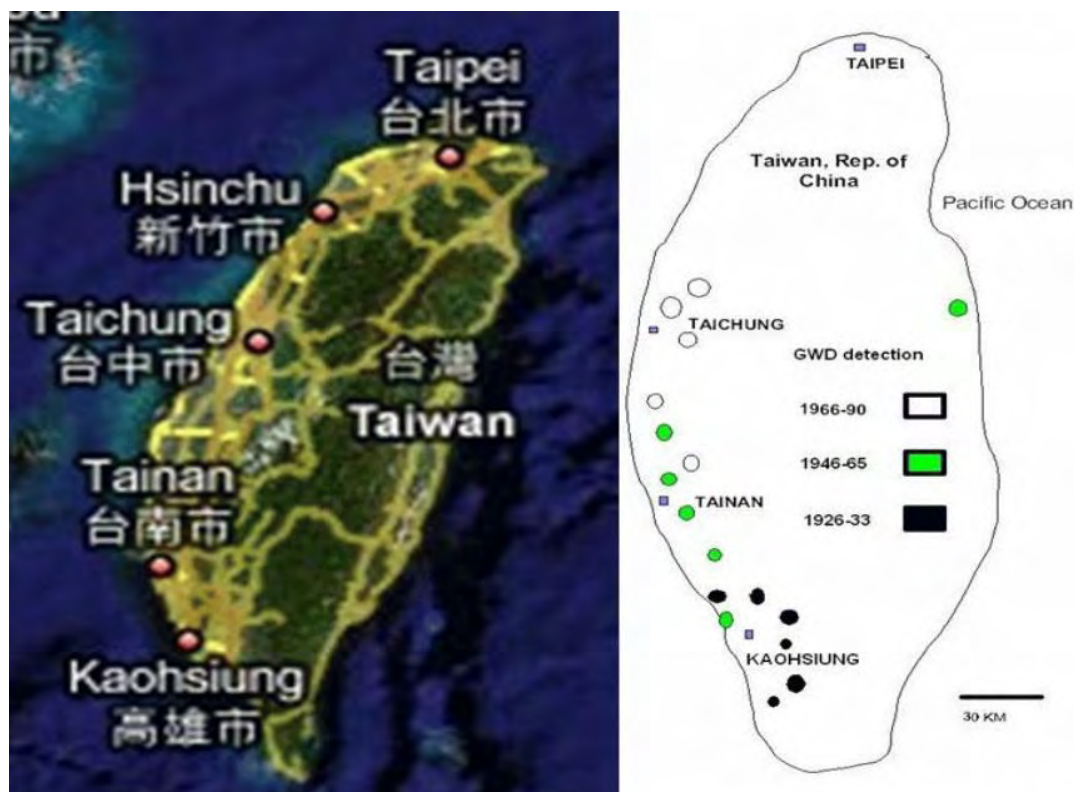
Figure 5.11. Distribution and chronology of the reported incidence of guava wilt disease in the Republic of China, Taiwan.

GWD was reported in the Philippines by Quimio *et al*. (1984), but only 60 years after its recognition in Taiwan. What is surprising is that the known distribution of GWD in Asia is so limited, given the long history of GWD in the Republic of China of over eighty years. The historical agricultural policies of the Japanese colonial government in Taiwan (Myers and Peattie, 1984) during the period that GWD was discovered in that country (by the Japanese researcher, E. Kurosawa) strongly promoted agriculture development and new crops. Extensive movement of plant material occurred between different Japanese colonies during their occupation of those countries. One can presume that movement of guava propagation material also occurred in south East Asia at that time, as it still does today. The anomaly is that GWD did not spread faster during this period (or possibly present but not

151

reported until much later), and to other Asian countries where guava was widely grown. This is probably because clonally propagated orchards did not develop until after the Second World War. Open pollinated, small scale orchards would have presented a barrier to the spread of the disease on a large scale. Only comparatively recently have defined phytosanitary policies been implemented between sovereign countries (Abdallah and Black, 1998)

Since its first appearance in South Africa in 1981, GWD has spread to all the major commercial production regions (Fig. 5.2), over a period of approximately ten years, with the exception of the Cape guava production regions. Observations in areas recently infected with GWD have indicated that these new sites were mostly located in close proximity to major road routes (Fig. 5.2). In orchards, a similar trend was observed in that the first detection of GWD in an orchard was normally found adjacent to a farm access road (Fig. 5.3).

The spread of GWD in an orchard was non-random (Table 5.3), spreading from tree to tree primarily in tree rows but also across tree rows (Figs. 5.4-5.7). This form of non-random disease movement suggests high frequencies of root transmission (Kentz and Riker, 1951) and it is postulated that this is the major pathways of "in-orchard" spread of GWD. Rates of mature tree death would also support this hypothesis, where in the field, the onset of symptoms to tree death is usually very rapid (+/- 2-6 weeks depending on season), whereas pruning wound infected trees can take several months to succumb. Root grafting in older orchards was commonly observed. The lack of root anastomosis and minimal pruning wounds (due to age) in Orchard 3 probably contributed to the lower tree mortality rate.

Young orchards were found to initially have lower rates of disease spread. However, by the fourth or fifth year tree death rates had reached parity with older orchards. The greater the orchard density, the earlier the tree death rate reached that of older orchards and this may be related to the greater frequency of root grafting in older orchards as well as orchards of higher plant densities. In warmer localities, disease spread was faster and this correlates well with the growth temperature preferences exhibited by *N. psidii* (Chapter 4, Figs. 4.1, 4.3 and 4.4). *N. psidii* did not grow below 8°C.

The progress of GWD, in terms of the daily tree mortality rates per ha were closely related to ambient temperature (Fig. 5.8). Summer daily tree mortality rates were at least double those in the winter. In some of the hottest regions (such as Orchard 1 in Malelane), tree mortality rates over the season as high as 1.6 trees.ha^{-1}.day^{-1} were recorded (Table 5.3). However, during mid to late summer (January-February), daily tree mortality rates in excess of 1 tree.ha^{-1}.day^{-1} in heavily diseased orchards were often observed (Fig. 5.8). However, in the hotter production regions of the Lowveld (e.g. Kopoort, RSA and Moamba, Mozambique), tree mortality rates of 2-3 trees.ha^{-1}.day^{-1} have been observed (Grech, unpublished). These findings are consistent with earlier findings (Chapter 2), where the optimal growth temperature for *N. psidii* was found to be approximately 30°C (35°C for conidial germination). Lowveld summer temperatures in full sun frequently exceeded in 40°C in many of the guava orchards examined, but generally in the tree canopy, temperatures usually were found to be in the mid-thirties. *N. psidii* grows slowly at temperatures below 15°C, and therefore winter temperatures were primarily responsible for the noticeable retardation of the winter mean daily tree mortality rates compared to summer

mortality rates.

The volumetric spore trapping and recovery methods employed in this study resulted in the recovery of aerial propagules of *N. psidii,* albeit at low levels. Volumetric spore trapping methods showed a distinct daily rhythmicity in total spore recovery patterns (Fig. 5.9). *N. psidii* conidia were not discernable from many other conidia recovered on the collection tape. However, many conidia resembling Type 1 conidia (which are hydrophobic) were observed on the collection strips. These conidia would have been released from sporodochia on the aerial parts of the tree (Chapter 3, Fig. 3.16), specifically during daily periods of low relative humidity. The conidial release episode in Orchard 2 peaked between 09.00 and 15.00, in accordance with the findings of Gregory (1962) for other hyphomycetes. Rainfall completely inhibited conidial release (Fig. 5.9). These conidial release patterns are similar to those obtained for *Nalanthamala vermoesenii (Gliocladium vermoesenii*) in the wilting disease syndrome on canary palms in California (Feather, 1982). The aerobiology of *N. psidii* is believed to be similar in all seasons of the year, particularly in the hot guava production areas (such as in Orchards 1 and 2). Type 2 sporulation occurred throughout the year, as did tree death (albeit at lower levels in winter). In the cooler production areas (such as Orchard 5) conidial release was lower in the winter due to reduced tree mortality rates and diminished production of bark sporodochia. However, field observations by the author indicated that active conidial discharges as a result of rupturing of bark blisters occurred throughout the year. These findings are important because guavas are pruned heavily in late spring/early summer, a period when *N. psidii* conidial production was increasing. It is likely that contamination of pruning shears and other implements occurs during pruning periods,

154

leading to increased tree infection, as observed with other diseases (Mercer and Kirk, 1983; Northover, 1992; Agustí-Brisach *et al*., 2015).

The initial pre-deployment tests that were conducted on passive conidial collection onto open Petri dishes (Fig. 5.1) offered promise based on preliminary results that indicated acceptable conidial recovery efficiencies were possible (Table 5.4). Although large variations were apparent in these data, the method did successfully recover *N. psidii* conidia. In the field, the technique of exposed Petri dish conidial collection proved to be a failure for reasons not clearly apparent in this study. This method did not result in any recovery of *N. psidii.* The method is very labor intensive and resulted in the culturing of numerous saprophytic fungal contaminants such as *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp. and *Alternaria* spp. that rapidly colonized the exposed Petri dishes, even with the addition of fungal growth retardants in the media. This issue was not problematic in the pre-field deployment tests because there were fewer background saprophytic contaminants present. The lack of a selective or partially selective media for *N. psidii* during this study restricted further elucidation of the aerobiology of *N. psidii.*

Air turbulence in Orchard 2 was very low and this may have contributed to the low dispersion of Type 1 conidia from the disease front. Tropical summer storms invariably led to substantial tree damage and this may also provide infection courts for *N. psidii,* which is a wound pathogen.

Irrigation did not seem to be a major factor in disease spread, except in orchards that were flood irrigated, such as Orchard 2. In Orchard 2, the movement of GWD down the tree row

was faster than the upward movement by approximately a factor of two. Irrigation water was delivered to the top of this orchard and gravitated down the tree rows to drainage canals at the North West orchard corner at the bottom of the slope. GWD spread in this orchard seemed to follow the direction of the slope (Fig. 5.5). It is postulated that in flood irrigated orchards, movement of *N. psidii* propagules such as Type 2 conidia or chlamydospores may be a greater factor in GWD spread than in low volume, micro-irrigated orchards, where water delivery is localized to a particular tree station.

N. psidii was detected at low levels in irrigation water collected at the bottom of the flood irrigated Orchard 2 (Fig. 5.10), and it is postulated that these water borne propagules infect wounded tree roots and eventually colonize the tree. Although *N. psidii* was detected, the dispersive aquatic propagules could not be found. It is postulated that they were the Type 2 (hydrophilic) conidial type and/or chlamydospores. In flood irrigated orchards water was frequently found to pass through the basins of dead and dying trees and then into the basins of healthy trees. Movement of GWD in flood irrigated orchards was found to move more rapidly in the direction of the water flow (slope), even though root grafting between trees in the same tree row was common. Further characterization of the aquatic and aerial dispersal of this pathogen will be assisted by the development of a semi-selective medium for *N. psidii* (Hong *et al.*, 2013). Chlorination of micro-irrigation systems utilizing surface waters potentially contaminated with *N. psidii*, has been found previously to eliminate infective propagules (Grech and Rijkenberg, 1991).

Sporulation of Type 2 conidia were found on infected roots as sporodochial masses,

frequently found in a calcium rich aggregates (Chapter 6, Fig. 6.13), and these were generally abundant under the leaf litter of dead and dying trees. It is postulated that these propagules are the major aquatic and subterranean infective propagules, due in part to their hydrophilicity. Many wounding agents are commonly found in the soil (Bruehl, 1987) that can provide the necessary wounds for tissue colonization by *N. psidii*. Although the methods used for spore collection from irrigation water were not entirely satisfactory, as reflected in the low recoveries achieved (Fig. 5.10), these data did indicate seasonal fluctuations of spore loads in irrigation water. This information may be of importance in the control of this disease in flood irrigated orchards if it can be shown in future studies that fungal inoculum loading is indeed less in all seasons except in the summer rainy season.

In these studies, however, the variation in numbers of propagules of *N. psidii* recovered from water samples was thought to be due to several factors, including fluctuations in the water quality between irrigation samples, and the tendency of propagules to aggregate on organic matter in clumps (Harrison and Lowe, 1987). A further difficulty was in identifying colonies of *N. psidii* in the Petri dishes before overgrowth by other fungi occurred. The development of a selective media for the isolation of aquatic propagules of fungi in the genus *Nalanthamala* will substantially alleviate this problem for future studies. Generally background contamination was higher in the samples with higher levels of suspended matter. It is thought that the many conidia were probably attached to suspended organic matter, which was then removed in the filtration process. Overall, however, these studies clearly showed the dispersive ability of *N. psidii* in water and in air.

Root anastomosis in Orchard 5 was common but the experiment test trees in this treatment were far (+/- 50 m) from the disease front and each 4 tree replicate was assessed on the incidence or absence of GWD, and not the degree of infection. Additionally, aerial infection often resulted in sectorial infection courts that were easily identified as a pruning wound (Chapter 6, Figs. 6.10 and 6.11). Further verification of the existence of these aerial infection courts was made by paring back the bark and wood to assess tissue discoloration. This usually confirmed the development of a pruning wound infection. Although the assessment of these pruning treatments occurred at 6 month interval, orchards were visited on a monthly basis. Trees were observed for infection and specifically, for the presence of aerial infection courts as a result of pruning. From these studies, pruning wound infection represents an infection route for the pathogen that greatly expands its dispersive potential and is likely to be the primary mechanism in the establishment of new infection centers within and between orchards. However, as statistically described earlier, once GWD is established in older orchards, the putative main mode of tree to tree transmission is via root grafting, which is common in older orchards. Root graft disease transmission is well documented in tree to tree spread of vascular wilting fungi (Stipes and Campana, 1981). In older orchards it is hypothesized that tree to tree infection via root grafting would mask the effects of pruning on the spread of GWD in active disease loci. However, it would seem from this research that pruning wound infections have the potential to exacerbate the spread of GWD in young (<7 years) orchards (low or no root grafting), as well as being an introduction route into new unaffected orchards.

In Taiwan guavas are pruned frequently, to not only reduce and maintain low canopy

heights, but also to modify crop harvest schedules. These multiple pruning practices in Taiwan do not appear to exacerbate GWD (Grech, 1988) to the same degree as in South Africa. This may be due to higher levels of tolerance to GWD in the cultivars used in Taiwan compared to South Africa. Historically, Taiwan has selected guavas for disease tolerance, primarily to aerial infection as this was, until recently (Hong *et al*., 2013), presumed to be the major infective pathway in commercial guava orchards in Taiwan. An important factor in the interpretation of these results on pruning frequency and the incidence of GWD is to consider that commercial pruning operations do not normally employ sterilization of pruning tools as a standard practice. As such, the spread of the fungus may occur on field implements during pruning, as in other fungal epidemics (Agustí-Brisach *et al*., 2015) such as pink rot of palm trees caused by *Nalanthamala vermoesenii* (*Gliocladium vermoesenii*), as described by Feather (1982).

The sharp rise in the daily tree mortality rate in October (Fig. 5.8) could have been due to the annual orchard pruning that occurs during early September, in conjunction with higher temperatures that are conducive to the growth of *N. psidii.* In view of this potential mode of disease spread, proactive management measures should take cognizance of the importance of tool sterilization between trees, and the need for protectant fungicidal or biocontrol sprays immediately after pruning. This is particularly important where GWD is already present in an orchard or if the disease is present in the vicinity.

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CHAPTER 6: GUAVA WILT DISEASE (GWD) SYMPTOM DEVELOPMENT UNDER CONTROLLED AND NATURAL CONDITIONS; AND THE DEVLOPMENT OF A DISEASE RATING SYSTEM FOR GWD

ABSTRACT

Several experimental sites were chosen in three countries to monitor the disease and plant condition. Two clonal cultivars were utilized (Fan Retief and Laia-Pai), as well as seedlings derived from open pollinated Fan Retief seeds. Naturally infected trees and plants artificially inoculated with *N. psidii* were used to investigate the dynamics of development of GWD. Symptom expression was most rapid with trees with their main trunks inoculated with the pathogen. From the onset of symptoms to tree death in field trees, it typically required 2-3 weeks in summer and 4-5 weeks in winter. Controlled environment inoculations of the main tree trunk, at a constant greenhouse temperature of 28°C resulted in the first symptoms appearing at 10 weeks followed by a progression to tree death at 16 weeks with the GWD-susceptible Fan Retief variety. Higher temperatures accelerated this process. Profuse bark sporulation was usually evident by Week 12. The moderately resistant Laia-Pai expressed first symptoms at Week 21 and died by Week 37. Pruning wound inoculated Fan Retief greenhouse trees expressed symptoms within 1-2 weeks following inoculation but remained alive for on average 24-35 weeks, a period significantly longer than the trunk inoculated plants. None of the moderately resistant Laia-Pai trees that were pruning wound inoculated died. The sequence of major symptom expression in trunk inoculated guava trees commenced with leaf reddening followed by sectorial wilting, fruit drop, active xylem (sapwood) discoloration, emergence of bark sporodochia, bark discoloration, unilateral canopy collapse shortly followed by plant death. These symptoms and events were categorized into a disease rating scale comprising of 6 ratings and identified as the Guava Wilt Disease Rating Scale (GWDRS).

6.1   INTRODUCTION

GWD caused by *Nalanthamala psidii* (Biourge) Schroers, has been responsible for the destruction of hundreds of thousands of trees in Asia and Africa (Hsieh *et al*., 1976; Grech, 1985 and 1987). The first recorded discovery of GWD was in the Republic of China, Taiwan in 1923 (Kurosawa, 1926) and is known there as Li-ku-Bin. To date, the disease is limited to the tropical and sub-tropical regions of Africa and Asia. Globally, in all production areas where the disease has been recorded, it has been found to infect all commercial cultivars to varying degrees, depending on genetic resistance to this fungal pathogen. GWD has also been observed to show seasonality in regard to symptom expression (Leu and Kao, 1979; Grech, 1982 and 1983; Schoeman *et al*., 2012), although symptoms may appear at any time throughout the growing season.

The reliance of a horticultural industry on only one or a few cultivars of a fruit crop can have catastrophic consequences, as experienced in the banana industries of Central and South America during the late nineteenth and first half of the twentieth century, when Panama disease first made its appearance and subsequently destroyed over 100,000 hectares of bananas in central America of the then popular Gros Michel variety (Stover and Simmonds, 1987). The introduction of the resistant Cavendish varieties solved the problem in Central and South America. Unfortunately a new race of the causal fungus of Panama disease, *Fusarium oxysporum* f.sp. *cubense* (Race 4) has arisen in South East Asia, Australia and Southern Africa that attacks the Cavendish varieties (Su, *et al*., 1986).

The aims of this research were to clearly identify and record the progression of symptoms

164

of GWD under controlled conditions as well in field trees. From these observations, a disease rating scale was constructed in order to provide an assessment tool for the precise identification of the disease stage and symptom progression.

6.2   MATERIALS AND METHODS

6.2.1 Symptomatology

All experimental sites were infected with *N. psidii*, and the disease incidence at the onset of these experiments was not greater than 25% tree death in any one orchard. The various orchards had certain differences in cultural conditions (Table 6.1) and the experiments conducted at these sites were executed over several years and were not all run simultaneously. The 'Fan Retief' clonal guava selection was used at most sites except at Ta Sir, Taiwan, where all the plants were of the Laia-Pa cultivar, and at Malkerns, Swaziland where open pollinated seedlings from Fan Retief parents were used. Healthy field trees were also inoculated in the field by the trunk inoculation method described in Chapter 2, which comprises of cutting a 1 cm^2 bark flap in the main trunk, and placing 1 ml of a *N. psidii* conidial suspension, adjusted to approximately 5 x 10^5 to 2 x 10^7 conidia.ml^{-1} (with 0.1% Tween^{20} added). Inoculation wounds were covered with first Parafilm$^®$ and then masking tape.

Table 6.1. Characteristics of eight guava orchards affected with GWD

Orchard Location	Orchard characteristics					
	Age	Ctv[a]	Irrig[b]	Plts/ha	Pruning cycle/yr	Initial GWD %[c]
1. Malelane	27	FR	ME	400	1	0.3
2. Louw's Creek	22	FR	F	400	1	10
3. Louw's Creek	4	FR	F	600	0	3
4. Hazy-view	18	FR	US	400	1	11
5. Bron-dal	20	FR	OS	500	1	10
6. Taisir (Taiwan)	8	LP	D	800	4	17
7. Nelspruit	28	FR	F	400	1	25
8. Malkerns (Swazi-land)	12	SL	D	2000	0	19

[a] Cultivar; FR= Fan Retief, LP= Laia-Pa, SL= seedlings. [b] Irrigation delivery method; F = flood, US = under canopy sprinkler, OS = over canopy sprinkler, D = dryland, ME = micro emitter.
[c] Initial infection level at the first orchard inspection (total number of trees infected or dead as a result of GWD expressed as a percentage of the total number of trees in the orchard).

The eight experimental orchards had a variety of irrigation systems ranging from furrow-flood to low volume drip systems (Table 6.1). All orchards were flat to gently sloped except for Orchard 2, which had a pronounced slope of approximately 7° to the horizontal plane. The slope ran downhill from the North West to the South East.

6.2.2 Visual symptom expression

Field trees at various disease stages were closely observed and their symptoms were described. In certain orchards (No 1-7, Table 6.1), healthy trees were selected in close proximity (+/- 20-50m) to diseased trees and monitored over several months during which time they contracted GWD. From the onset of symptoms until tree death, disease symptoms were recorded and photographed. Several categories of disease stages were defined and a disease key developed, as described in Table 6.2.

Two sets of three, 3 year old containerized guava plants (cv. Fan Retief and Laia-Pai) were placed in a temperature controlled greenhouse ($28^{O}C$) under ambient light conditions. Trees were placed under drip irrigation utilizing a single $1L.hr^{-1}$ dripper per tree (Netafim, Kraaifontein, RSA). Drainage water was led away from the base by the use of closed drainage channels that discharged drainage water outside the greenhouses in such a way that it did not contact any other pot. Tree containers were arranged next to one another, 50 cm apart, in a row. The main stem of the center tree was inoculated as described above, utilizing the bark flap method. The two other trees in each set were uninoculated. Inoculated and uninoculated trees were pruned two weeks prior to inoculation and the uninoculated trees again, at the onset of bark sporulation on the inoculated tree. Trees were then left unpruned for the duration of the rest of the experiment. Symptoms on these trees were recorded over several months. Tissue isolations (as described in Chapter 2) to recover *N. psidii* were conducted on the greenhouse inoculated and naturally infected trees at the onset of symptom expression. Two additional trees of each clonal variety were also pruning

wound inoculated using a conidial suspension as described above, applied to a freshly cut wound and covered.

6.3   DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, treatment means were separated using the Waller-Duncan k-ratio t test (Little and Hills 1990).

6.4   RESULTS

6.4.1 Symptomatology

The symptoms of GWD were similar in all sites that were monitored (Figs. 6.1-6.2), although rates of tree death and disease spread varied between orchards (Chapter 5, Table 5.3).
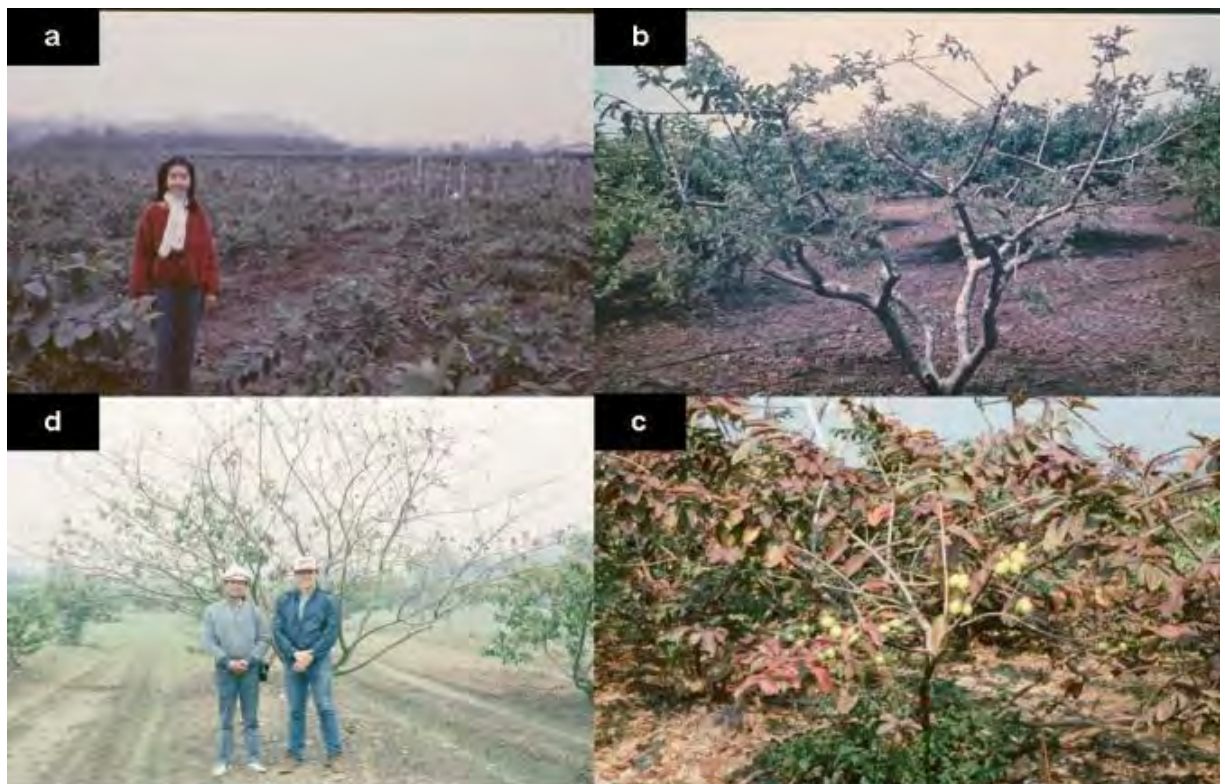
Figure 6.1. Cultivation of guava, and symptoms of guava wilt disease, in the Republic of China, Taiwan: a, typical guava cultivation in Taiwan in which the tree canopy is kept small; b, early stage GWD symptoms; c, typical GWD early symptom expression, note the leaf reddening. d, a variety at the Fengshan Agricultural Research Station where guava selections are screened for tolerance to GWD.

Figure 6.2. Symptoms of guava wilt disease in Southern Africa: a, adjacent trees exhibiting (from left to right) various canopy symptoms associated with GWD (dead, early canopy decline and a healthy tree); b, dead tree on the left, healthy tree right; c, rapid sectorial canopy collapse, d, onset of the GWD symptoms in a previously unaffected orchard, typically initiating at the orchard perimeter along an access road.

Trunk inoculated greenhouse Fan Retief trees exhibited symptoms (leaf reddening, terminal leaf wilt) at approximately ten weeks after inoculation and generally died by Week 16-20 (Fig. 6.3). Initial leaf wilting was found to occur as both an apical leaf wilt as well as a sub apical leaf wilt. Sporulation generally was evident within 8-10 weeks after trunk inoculation (Fig. 6.3). The moderately resistant Laia-Pai expressed first symptoms at week 21 and died by week 37. No evidence of pruning wound infected tree mortality was evident

in the Laia-Pai variety.
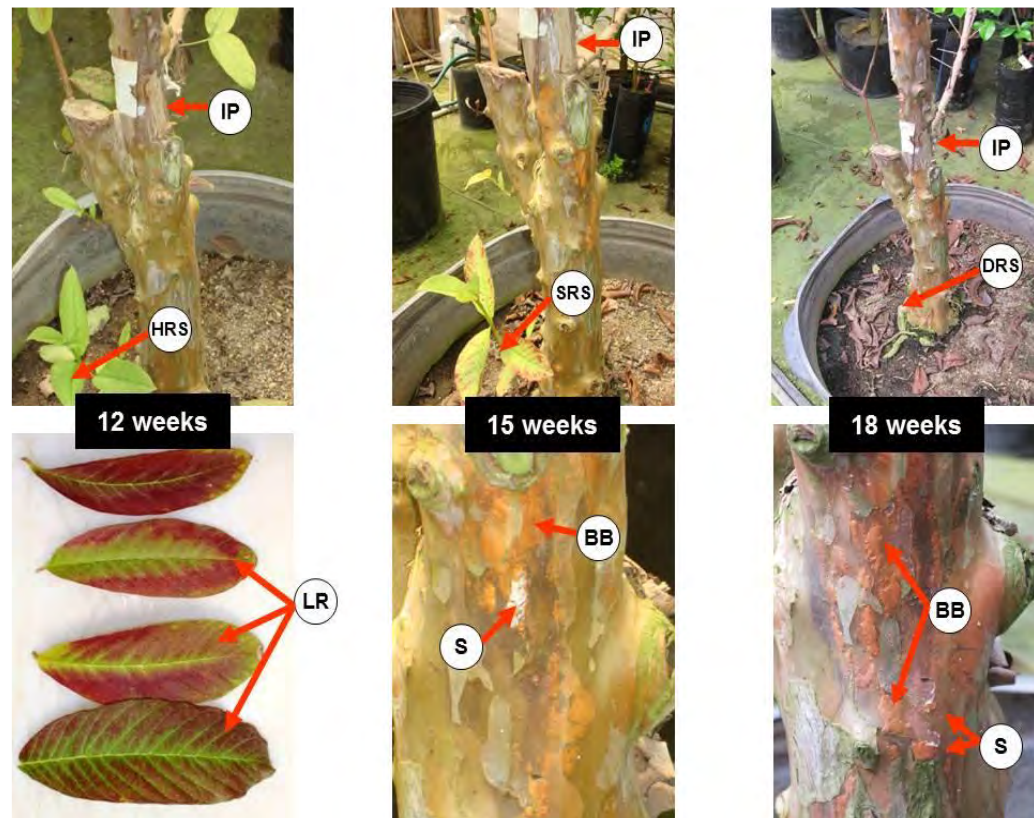
Fig. 6.3. GWD symptom expression in a greenhouse grown clonal guava seedling inoculated with *N. psidii* and observed over an 18 week time period. 12 weeks: the root sucker below the inoculation point is symptomless, red pigmentation expression on leaves from terminal branches. 15 weeks: defoliation and leaf reddening on the root sucker, bark blisters and sporodochia evident on the main trunk. 18 weeks: root sucker has died, bark reddening, extensive bark sporulation on main trunk. BB = bark blisters; IP = inoculation point; HRS = healthy root sucker; SRS = symptomatic root sucker; DRS = Dead root sucker; LR = Leaf reddening; S = Sporodochia.

Infected guava vascular tissues generally became discolored with advancing disease symptoms, with xylem tissue color being the most affected by GWD (Fig. 6.4).
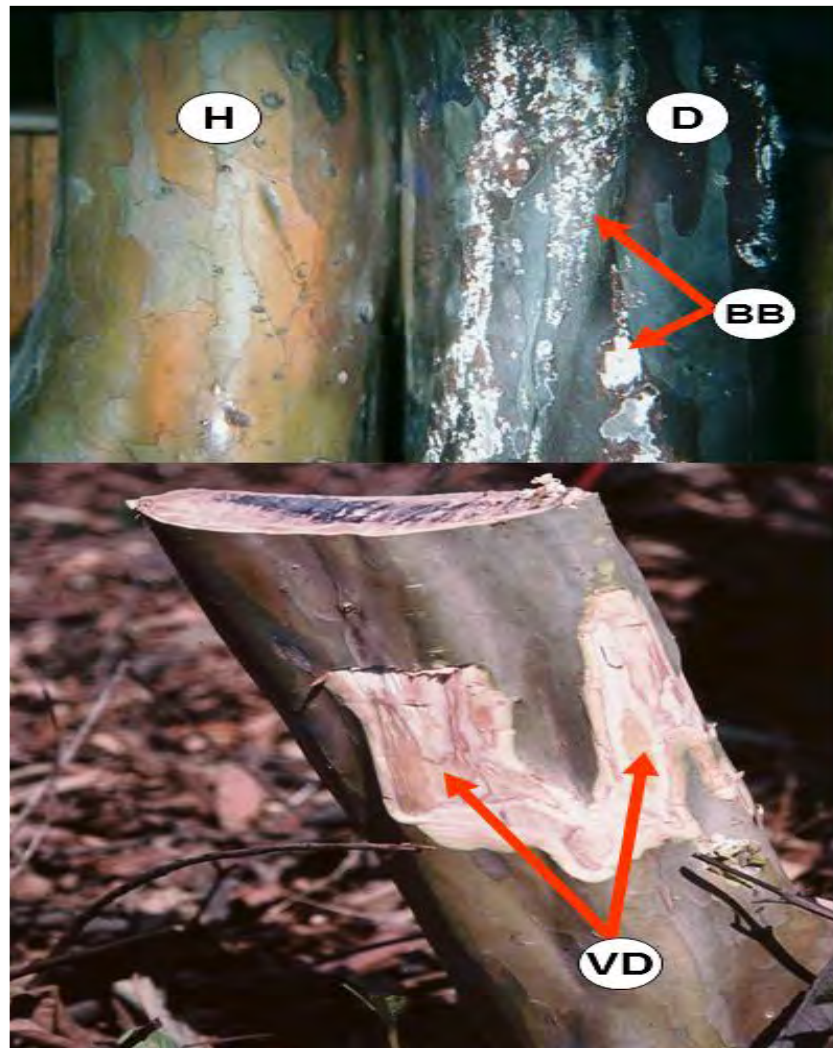
Figure 6.4. Healthy and diseased guava trunk sections. Top; healthy left, diseased right showing bark discoloration and ruptured bark blisters exposing sporodochia. Bottom; *in situ* GWD infected tree stump with pronounced wood discoloration beneath the tree bark. H = Healthy. D = Diseased. BB = Bark blisters. VD = Vascular discoloration.

Invariably, *N. psidii* could be isolated from the discolored tissues (Figs. 6.4, 6.3, 6.8).

Interestingly, in some cases, at the early stages of leaf wilt (Figs. 6.6 and 6.7). *N. psidii* could not be isolated from peripheral canopy stem tissues where the leaves were attached.

Adjacent trees that were pruned after the onset of sporulation in the inoculated center tree became infected from pruned terminal branches (Fig. 6.5).
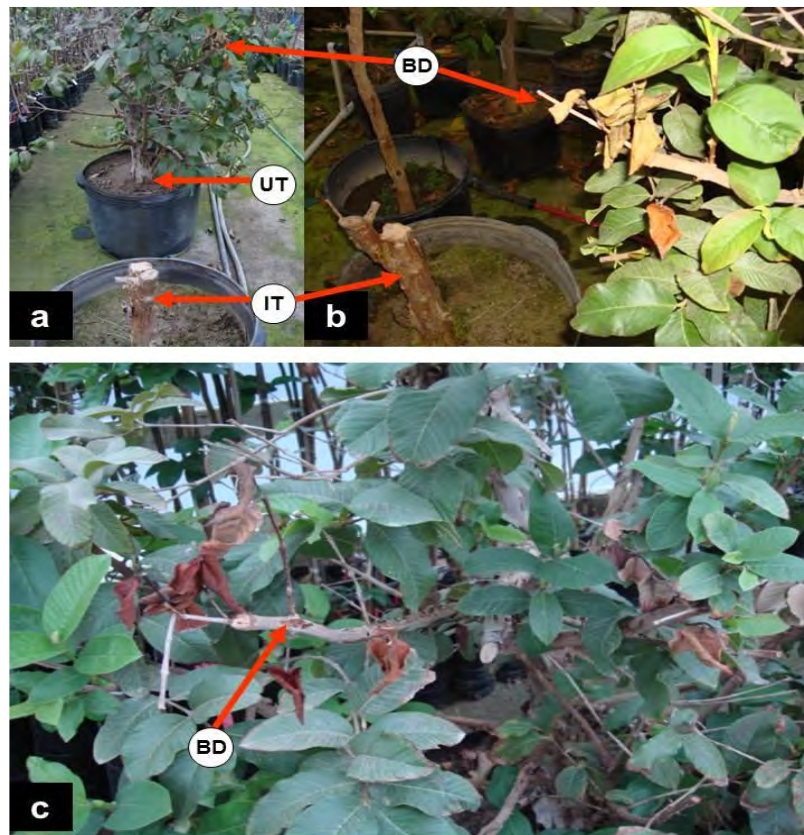
Fig. 6.5. GWD symptom expression in greenhouse inoculated plants: a, an uninoculated (UT) clonal guava seedling adjacent to a guava seedling previously inoculated (IT) with *N. psidii* (and that had subsequently died); b, an uninoculated control (UT) tree adjacent to a deceased tree with (active sporulation) showing symptoms of GWD on a pruned branch; c, close up of symptoms of branch dieback (BD) on the pruned limb (as b), are evident. Note that these adjacent plants had no root or soil contact.

These trees remained alive for longer periods than the trunk inoculated trees (15-18 weeks vs 24-35 weeks), although symptoms invariably were evident sooner (Fig. 6.5), usually within 1-2 weeks post inoculation. Trees that were inoculated in their main trunk frequently produced root suckers from the base of the main stem. These suckers were observed initially to remain generally healthy even as the main canopy became symptomatic for GWD (Fig.

6.3). Trees that succumbed to pruning wound infection were also observed to produce root

suckers. These suckers also remained healthy for some time after the main trunk had died

but eventually became symptomatic (Figs. 6.6 and 6.7). Canopy GWD symptom expression

in these root suckers was often sectorial and in some cases *N. psidii* could not be isolated

from afflicted branch sub-apical and apical termini (Figs. 6.6 and 6.7).

Fig. 6.6. GWD symptom expression in a root sucker emanating from a greenhouse grown guava seedling naturally infected with N. *psidii* through a pruning wound in the main canopy of the tree. The main trunk died and was removed immediately prior to Photograph 'a' being taken: a, T = 0; b, T = 8 days; c, T = 13 days; d, T = 19 days; e, T = 23 days; f, T = 25 days.
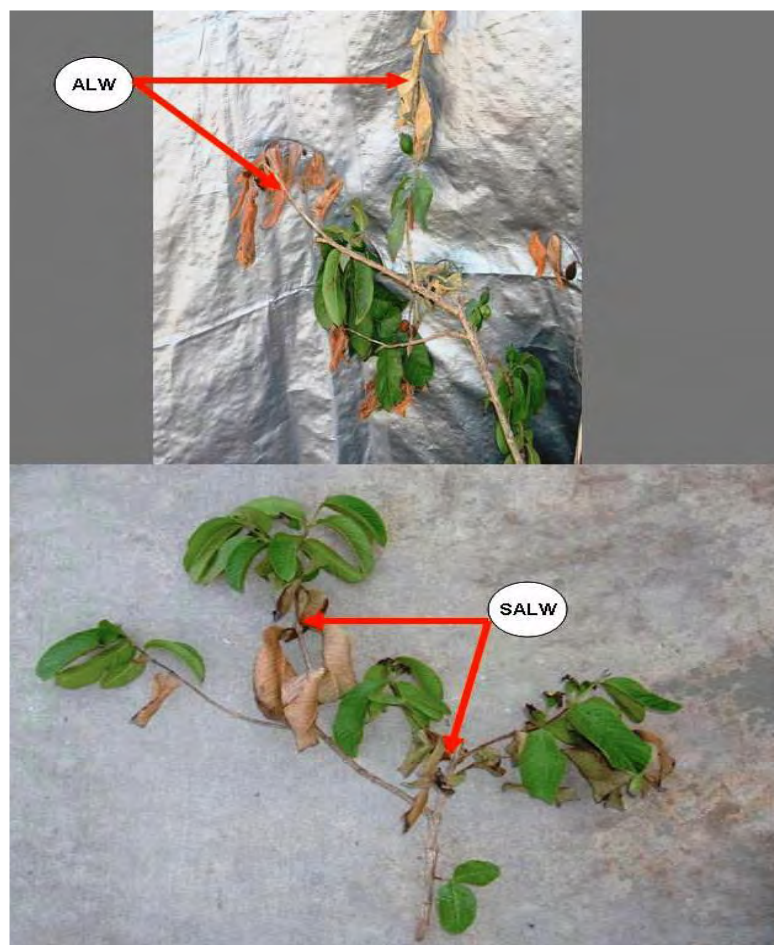
Fig. 6.7. Apical leaf wilting (ALW) and sub-apical leaf wilting (SALW) in branches from a GWD infected guava tree root sucker (as in Fig. 6.6). *N. psidii* could not be isolated from the branch tissues where the leaves had wilted.

6.4.2 Disease Rating Categories

Six disease categories were developed for GWD (Table 6.2) based on visual symptoms

(Figs. 6.10- 6.13).

Table 6.2. Guava wilt disease rating categories

_____

Rating category	Visual Symptoms
0	Healthy (Fig. 6.10a).
1	Slight paling of canopy followed by a reddening of the foliage (Figs. 6.1c, 6.3, and 6.10b). Leaves and fruit show an increased susceptibility to anthracnose. Fruit respiration rate increases leading to increased ripening. Flagging of foliage and sectorial wilting of branches, extending to other branches (Fig. 6.10b).
2	Pronounced sectorial wilting of the canopy (Fig. 6.11a). Partial defoliation of affected branches with many desiccated leaves retained. In autumn and winter, trees affected by GWD may appear to be in a state of stasis in terms of disease symptoms, but as warmer temperatures return, the tree decline continues unabated. Disease spread in the field is non-random, usually spreading from tree to tree (Fig. 6.13).
3	Unilateral canopy wilting (Figs. 6.11b and 6.13b) Immature fruit largely abscise, while more mature fruit stay attached and mummify, imparting an impression that the tree has been fire scorched. The bark and wood become darker (Figs. 6.3 and 6.4) and pink dry spore masses erupt from sporodochia below bark blisters (Figs. 6.3 and 6.4). Sporodochia found on exposed dead roots as well as roots beneath the leaf litter (Figs. 6.8 and 6.9). Disease progression is maximal in the summer.
4	Very few leaves left on the canopy (Fig. 6.12a), depending on the season the tree may re-flush but shoots will die shortly after emergence. Tree death follows within several days. Bark universally discolored [a grey/reddish brown hue (Figs. 6.3-6.4)] accompanied by profuse aerial sporulation emanating from sporodochia.
5	Dead (Fig.6.12b).

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The development of the disease between Stage 0 (healthy) and Stage 1 (Fig. 6.10) generally

varied from one month to several months in the field. Transition from Stage 1 to Stage 2

(Figs. 6.10b and 6.11a) took approximately one month. From Stage 2 to Stage 3 was usually

rapid, lasting not longer than 2-3 weeks (Figs 6.11a and 6.11b). The transition from Stages 3 to 4 (Figs. 6.11b and 6.12a) was very fast (less than 2 weeks in the summer season), followed rapidly by complete tree decline and death [Stage 5 (Figs. 6.12b and 6.13)]. By comparison greenhouse trunk inoculated three year old trees declined over approximately 18-20 weeks (Fig. 6.3).

6.5   DISCUSSION

The major symptomatology of GWD is largely identical in Taiwan and South Africa (Figs. 6.1-6.2) and the minor differences between the two regions may be attributable to varietal differences. Controlled greenhouse inoculations (both natural and artificial) resulted in expression of symptoms that were identical to observed field symptoms.

The disease rating scale developed for GWD (Table 6.2) was useful in assessing and enumerating certain diagnostic techniques for GWD (water uptake and nutritional status). However, the rating scale is limited in its value in assessing economic loss and disease levels due to the rapidity of disease progress (particularly between Ratings 2 and 4). This is unlike the pythiacious root rots, which induce slow declines, and with which tree recovery is possible following appropriate chemical treatments (Le Roux *et al.*, 1991) and can be accurately quantified by a rating system.

Due to the rapidity of vascular wilting diseases such as GWD, recovery of infected trees in South Africa has not been observed. In vascular wilts, disease progression from the onset

of symptoms until plant death is generally rapid and recovery is rare (Bruehl, 1987). Occasionally a diseased plant was observed to stabilize during winter, often producing a weak post-pruning flush in spring. In some cases, root suckers emerged from diseased trees but as in the greenhouse trials, these root suckers (and eventually the entire tree), succumbed (Figs. 6.3 and 6.6). An interesting observation in greenhouse inoculated plants that correlated well to field tests and observations was that sub-apical and apical wilt and dieback of the canopy (Figs. 6.6 and 6.7) was not always associated with active fungal colonization. The putative cause of this phenomenon is believed to be the acropetal translocation of wilting toxins from lower tissues colonized by *N. psidii*. Xylem mobile wilting toxins have been extensively reported previously for other fungal vascular wilts of plants (Stoddart and Carr, 1966; Graniti, 1991) Studies not reported herein by Grech, have identified plant wilting toxins produced by the GWD pathogen, *N. psidii.* As discussed earlier, these situations were usually encountered where infection occurred via the roots. Many vascular wilting fungi produce toxins in advance of the hyphae (Stoddart and Carr, 1966). *N. psidii* produces extra cellular cellulases (Grech, unpublished), characteristic of many *Gliocladium* species (Morquer *et al.*, 1963), which are postulated to be involved in the development of the brown discoloration of the xylem tissues with advancing disease symptoms.

Previous studies not reported herein by Grech, indicate that the extent of tissue discoloration in infected tree specimens is directly proportional to the disease rating and most consistently evident in primary and secondary root tissues. These observations are broadly in agreement with those of previous reports from the Philippines (Quimio *et al.*,

1984), Thailand (Athipunyakom and Manoch, 1998; Athipunyakom and Luangsaard, 2008) and India (Misra, 2006; Misra 2007). In these reports, distinct discoloration of the main roots and trunk woody tissues was described. Xylem tissue discoloration is also symptomatic of persimmon wilt, where brown to black wood discoloration occurs often before any canopy symptoms become apparent (Wilson, 1963; McRitchie, 1979)

In addition, varieties and/or selections that exhibited some tolerance to *N. psidii* as compared to the 'Fan Retief' selection showed greater and prolonged sectorial wilting. In all these cases, however, trees were observed to eventually progress to the complete canopy wilting stage, which occurred most rapidly during the summer months. Interestingly, terminal leaf reddening was a consistent early symptom that was expressed in inoculated greenhouse trees (Fig.6.3) as well as in naturally infected field trees (Fig. 6.1). These symptoms have also been reported from Taiwan (Leu and Kao, 1979) and India (Misra, 2006 and 2007). Red pigmentation development in plants is often due to the presence of certain flavonoids (such as anthocyanin) pigments being produced in dermal leaf tissues. Anthocyanin expression in plants is often associated with stress factors such as cold, pathogen attack or mineral deficiencies (Strack, 1997).

In Taiwan, some cultivars have been screened for increased tolerance to *N. psidii* (horticulturally most of these cultivars are not suitable in terms of pulp color, size and sugar content, for the South African industry, but their genetic tolerance may be valuable for breeding work). Although most of these selections (such as Pei Pa and Laia-Pai) are still vulnerable to infection with *N. psidii* , the genetic tolerance is such that infections

(particularly of the peripheral aerial tissues) do not result in rapid canopy declines (as witnessed in the 'Fan Retief' selection), but rather a slower gradual decline. This tolerance would certainly contribute to less pruning wound infection, which is particularly important in Taiwan where pruning is conducted more frequently than in South Africa.

Sporulation was observed in the field on all aerial parts of diseased trees, particularly in the latter canopy rating stages (Rating 3 to 5, Table 6.2) of GWD development (Figs. 6.3-6.4). Bark sporulation was usually preceded by a reddening of the bark, and sporulation was observed to develop under the bark tissues in blister-like structures. These bark blisters would desiccate and rupture, exposing Type 1 conidial masses produced from sporodochia (Figs. 6.3-6.4). Aerial sporulation was limited to Type 1 conidia (Chapter 2). The sporodochial development, structure, conidial color and discharge characteristics reported here are very similar to the conidial masses produced by the persimmon wilt pathogen, *N. diospyri*, as described by McRitchie (1979).

Bark blisters (and the associated Type 1 conidia) were occasionally observed on primary roots (Fig. 6.8), where these were exposed to the air, usually due to cultivation or water erosion of the irrigation basin. In the field, Type 2 conidia were exclusively produced in subterranean tissues, usually arising in sporodochial masses from primary and secondary roots (Fig. 6.9). These sporodochial conidial masses were frequently associated with calcium rich deposits adhering to the root surface.

Figure 6.8. Primary roots from *N. psidii* infected guavas: a, discolored primary and secondary roots, b, ruptured root bark blisters exposing sporodochia on *N. psidii* infected primary roots exposed to the air. S = sporodochial structures.
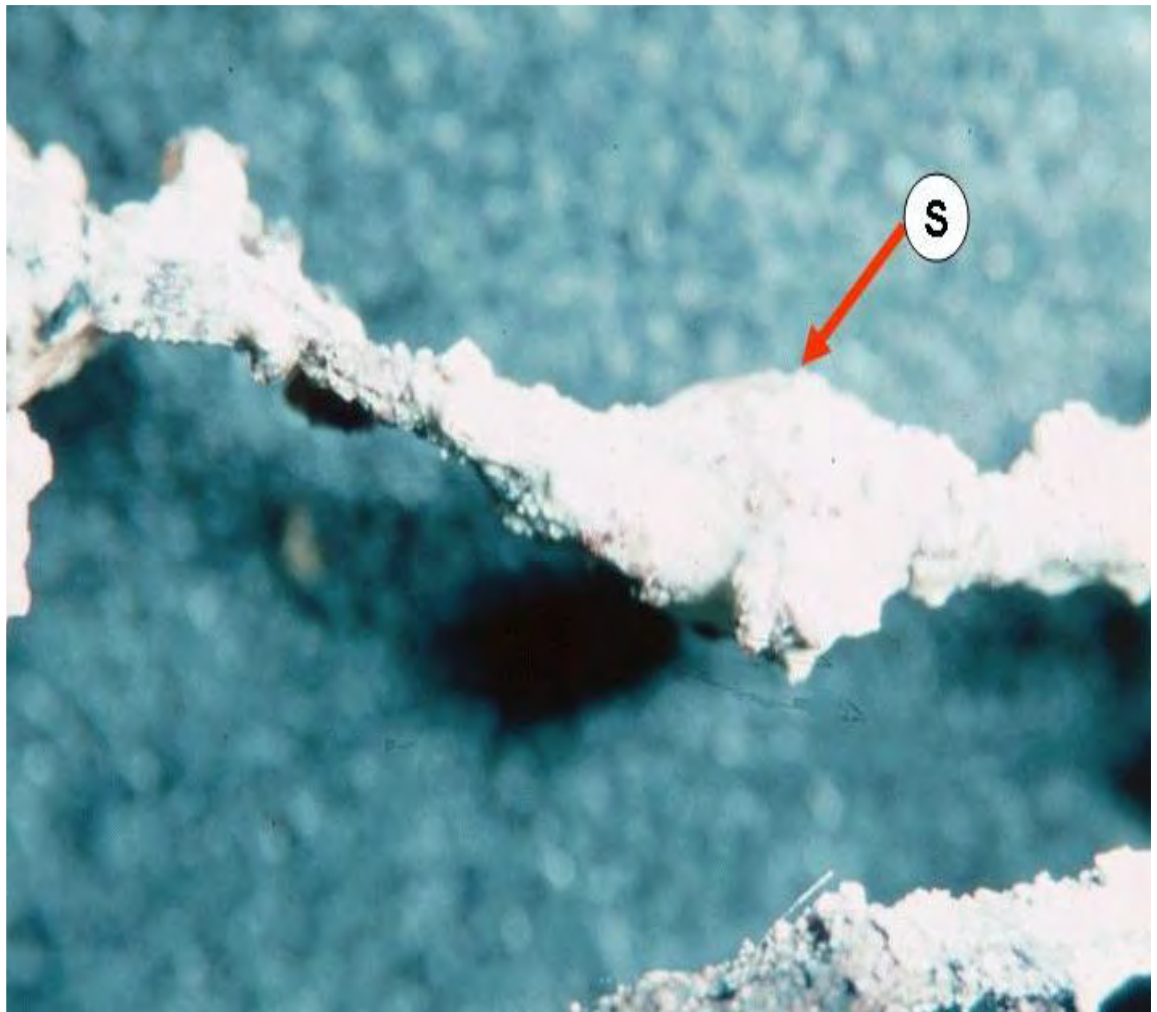
Figure 6.9. Feeder roots from an artificially inoculated clonal guava plant (cv Fan Retief) incased with white amorphous matter containing Type 2 conidia. S = white amorphous spore masses.

In these studies it was interesting that in the greenhouse trunk inoculations, of the Fan Retief cultivar, symptoms did not appear for at least 10 weeks. Practically this implies that apparently healthy field trees may be infected but simply are not expressive for GWD symptoms and therefore this is an important finding when developing management strategies for this disease, which should account for this latent period in tree removal programs. This is in contrast to aerial infection of pruning wounds where symptoms

develop more rapidly (1-2 weeks post inoculation) and hence easier for field diagnosis of GWD. The moderately resistant Taiwanese variety, Laia-Paia did not succumb to GWD when pruning wound inoculated but did when trunk inoculated, implying that wholly relying on aerial canopy challenges with *N. psidii* of guava plants in GWD resistance selection programs is a fallacious strategy.

The symptoms of GWD were found to be very consistent across numerous orchards and geography in Southern Africa. Disease expression in Taiwan generally was slower (due to higher levels of genetic resistance to *N. psidii* in commercial varieties), but the symptoms were identical with the exception of major tree to tree root transmission following orchard rows. This was primarily a result of much lower tree ages in Taiwan (due to GWD induced, frequent orchard replanting) and as such, much lower levels of root to root grafting.

From the onset of GWD symptom expression to tree death can be very rapid in the hotter guava production regions particularly during summer and in diseased orchards, daily tree mortality rates during these period can be as high as 2-3 trees.ha^{-1}.day^{-1}. Hence from the first expression of symptoms in a mature guava tree (particularly with root infection), the sequence of symptoms prior to tree death is rapid (Figs. 6.10-6.13), in some cases lasting no more than 1-2 weeks. The development of a Guava Wilt Disease Rating Scale (GWDRS) system for GWD has proven to be useful in subsequent studies where quantitation of the disease is desired, such as in seasonal epidemiology studies, prophylactic chemical disease suppression and disease resistance selection.
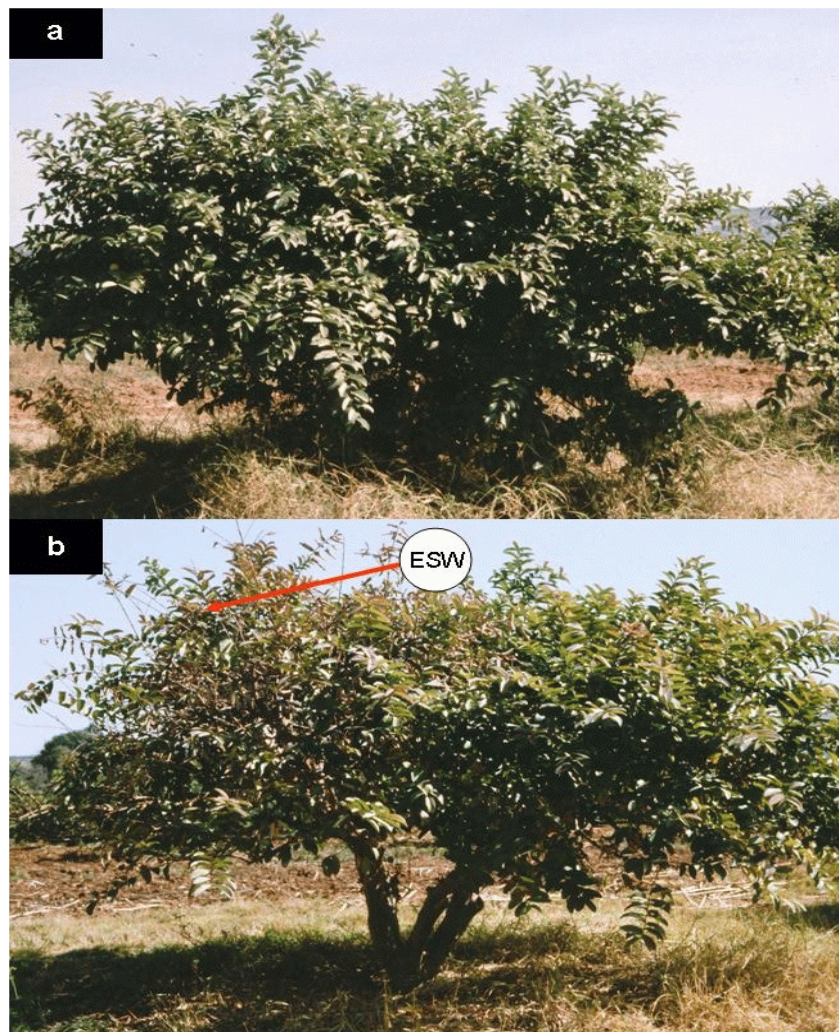
Figure 6.10. Stages and symptoms of guava wilt disease; a, Stage 0 = healthy; b, stage 1 = early disease onset with canopy discoloration and sectorial wilt visible.
ESW = Early sectorial wilt.
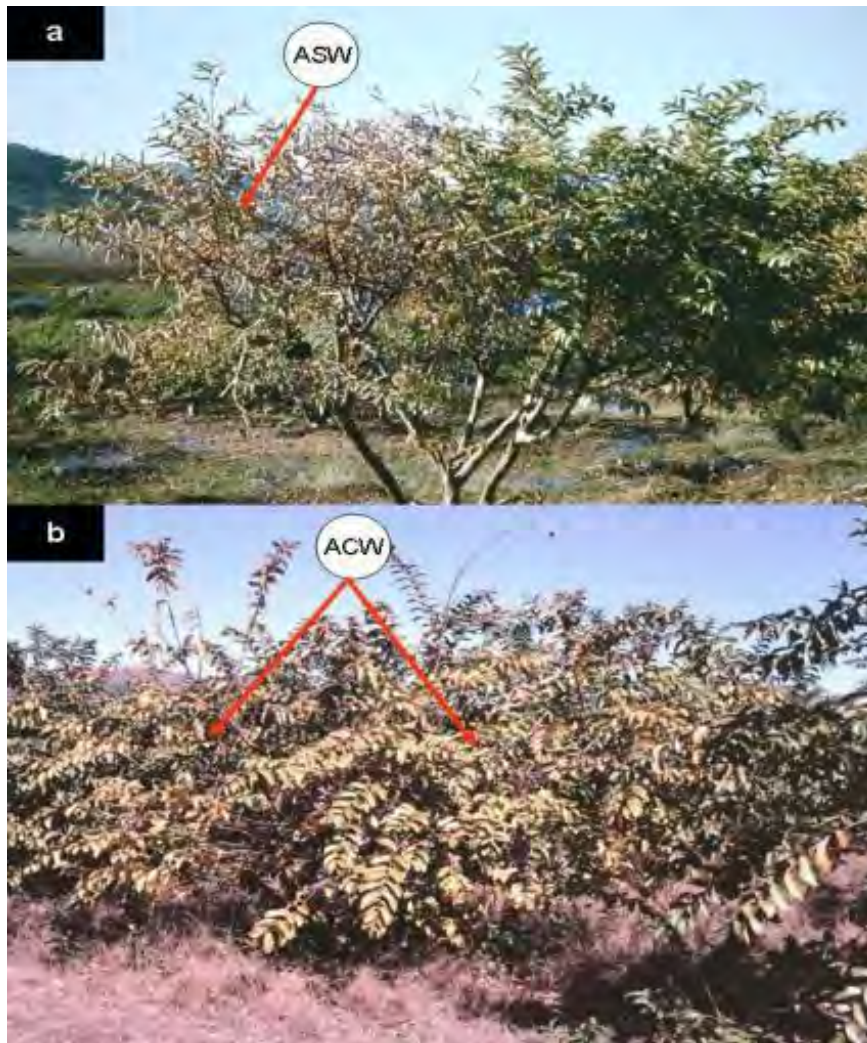
Figure 6.11. Stages and symptoms of guava wilt disease; a, stage 2 = pronounced sectorial canopy wilt, foliage discoloration, partial branch defoliation and mummified fruit; b, stage 3 = onset of overall canopy collapse, branch defoliation, foliage discoloration, fruit drop. ASW = Advanced sectorial wilt. ACW = Advanced canopy wilt.

Figure 6.12. Stages and symptoms of guava wilt disease: a, stage 4 = Overall canopy wilting, extensive canopy defoliation, bark discoloration, very limited regrowth and reduced flushing; b, stage 5 = dead, some desiccated and senescent leaves retained.
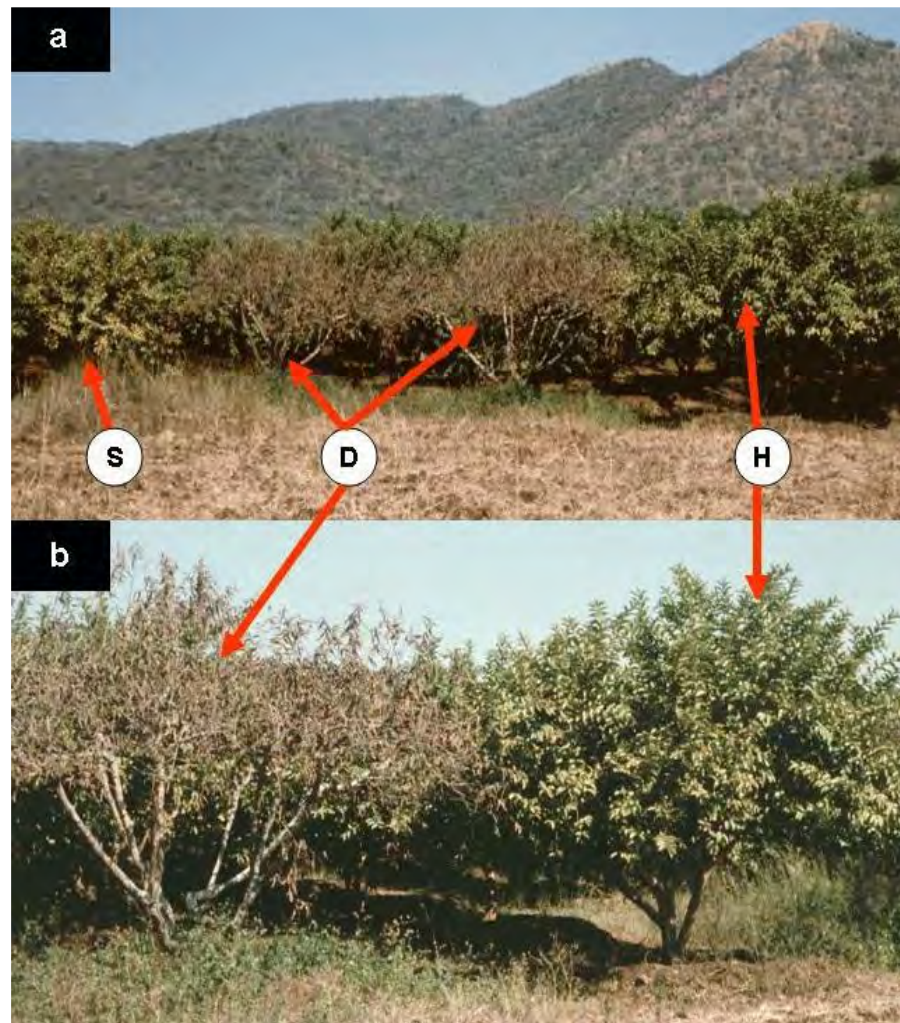
Figure 6.13. A guava orchard exhibiting various stages of guava wilt disease: a, trees at the advancing disease front (dead trees in the foreground have been removed) at various stages of decline (from left to right; early symptoms, dead and healthy); b, Left tree dead, right tree healthy. S = Symptoms. D = Dead. H = Healthy.

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CHAPTER 7: CONTROLLED GREENHOUSE AND LARGE CONTAINER-GROWN PLANT INOCULATION STUDIES ON VARIOUS PLANT TISSUES, THE HISTOPATHOLOGY OF *NALANTHAMALA PSIDII* IN HOST TISSUES, AERIAL SPREAD, AND *IN SITU* PROPAGULE LONGEVITY

ABSTRACT

In greenhouse and field studies, *Nalanthamala psidii* (Biourge) Schroers, a wound pathogen of guava (*Psidium guajava* L.), was found to be infective on all guava tissues tested, with the exception of the fruit pedicel (fruit terminus). These various tissue inoculations led to the development of guava wilt disease (and eventual plant death) with the exception of leaf inoculations (leaves became infected, and *N. psidii* could be recovered, but all infected leaves abscised prior to systemic plant colonization). This is the only known guava tissue that exhibits this hypersensitive response to *N. psidii* and in so doing averts plant death. Colonization of the active xylem (sapwood) tissues by *N. psidii* was three times faster acropetally (2.8 mm.day^{-1}) than basipetally (0.9 mm.day^{-1}) and as such aerial infection of pruning wounds generally induced tree death over a longer period of time. Primary or secondary root, trunk as well as secondary branch inoculation induced plant death most rapidly in the Fan Retief guava cultivar (+/- 2.5-3 months), followed by tertiary branch inoculation (+/- 4 months). Trees infected through pruning wounds were found to survive in some cases for over 12 months before succumbing to guava wilt disease (GWD). Tree mortality due to GWD was approximately three times more rapid in summer than in winter. The extent of vascular colonization by *N. psidii*, was well correlated to the Guava Wilt Disease Rating System (GWDRS) developed earlier in these studies. *N. psidii* was found to persist in dead guava tissues for approximately 12 months. *N. psidii* was found in culture to secrete cellulases.

7.1   INTRODUCTION

Nalanthamala psidii (Biourge) Schroers is a wound pathogen of guava (*Psidium guajava* L.) that induces a lethal wilting disease of the crop in two continental regions of the world (Leu and Kao, 1979; Grech, 1982, 1983, 1985; Quimio *et al*., 1984; Athipunyakom and Manoch, 1998; Schroers *et al*., 2005; Misra., 2006; Athipunyakom and Luangsa-ard, 2008; Schoeman *et al.,* 2012; Severn-Ellis *et al*., 2012; Hong *et al*., 2013, 2014). Guava wilt disease (GWD) is currently reported to occur only in Asia (Taiwan, Malaysia, the Philippines, Vietnam, Thailand and India[1]) and Southern Africa (South Africa, Mozambique, Swaziland and Zimbabwe). It is characterized by a very rapid canopy wilting syndrome. Vascular wilting pathogens are characterized by specialized mechanisms of transmission between susceptible hosts (Beckman, 1987, Yadeta and Thomma, 2013).

As there are no practical curative control methods for the control of vascular wilting diseases of plants beyond innate plant tolerance (Yadeta and Thomma, 2013), prevention is a primary component of disease management. An understanding of the possible disease transmission pathways of *N. psidii* is an important prerequisite for developing practical control measures. The aims of this study were to more fully assess the susceptibility of various plant tissues to *N. psidii* and the dynamics of vascular tissue colonization by this pathogen.

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[1] Misra, A.K., 2007, identified the causal organism of the guava wilt syndrome in India as *Gliocladium roseum*, which although unconfirmed, may be *N. psidii*.

7.2   MATERIALS AND METHODS

7.2.1   Greenhouse infection studies

Clonally propagated guavas (cv. Fan Retief) of approximately twelve months of age were produced from stem cuttings (as modified from the methodologies of Hafeez *et al*, 1988; Prasad *et al.*, 1988) and established in a sandy loam soil, in five liter pots and placed in a greenhouse. The plants were irrigated daily by hand. Fertilizer was applied according to the recommendations of Du Plessis and Koen, (1984); Koen, (1989); Koen *et al.,* (1990); Koen and Hobbs (1990) and De Villiers, (2004). The greenhouse was maintained at a constant temperature of 28°C, without supplemental lighting. Fifteen single plant replicates were used per treatment, in all greenhouse experiments (unless otherwise stated). In all greenhouse experiments, plants were arranged in either a randomized blocks design, or as a paired replicate design.

In all inoculation studies (unless otherwise stated), a spore suspension containing Type 1 and Type 2 conidia of *N. psidii* (Isolate CSF221) were used (Chapter 2). The spore concentration range in inoculation experiments was adjusted to 5.8×10^5 to 2.2×10^7 conidia.ml^{-1} (with 0.1% Tween^{20} added, unless otherwise stated). Plants were wound inoculated at selected anatomical positions (Table 9.1). Wounds were inflicted on the respective plant tissue by first lightly scraping the surface tissues, followed by three to five scalpel cuts, to a depth of 1.0 to 2.0 mm, approximately 5.0 to10.0 mm in length. In lignified tissues such as branches and roots, care was taken to ensure that the wounding procedure exposed xylem tissues. Wounded areas were sprayed with 1 ml of the conidial suspension and covered with Parafilm® (American National

Can, Greenwich, CT, USA). This inoculation method was used for leaf, twig, and root inoculations. Unwounded leaves and leaf pedicle surfaces were also inoculated as above, and covered with Parafilm®. Inoculation of the trunk and scaffold branches entailed cutting a 1 cm² bark flap, placing 1 ml of the inoculum in the flap and covering the wound with first Parafilm® and then masking tape. This method was also used for secondary roots, twigs and root suckers. In some cases the entire root or branch was cut through and the inoculum placed on the cut end, and covered as above. These terminal inoculations were also overlaid with tin foil.

Lesion development and leaf abscission were monitored daily in the leaf inoculation experiments. Similarly in the branch and lateral root inoculations, movement of the pathogen in the wood was recorded every three days by making small (<3 to5 mm) incisions into the xylem with a sterile scalpel and recording wood discoloration. If discoloration was found, small pieces of the wood were plated onto WAA, incubated at 30°C, and examined after three days for the presence of *N. psidii*. After observation and tissue removal, these observation wounds were painted with a 1% suspension of a contact fungicide (copper oxychloride), to prevent further infection through these wounds. Additionally, basipetal movement of the pathogen was monitored by observing wilting in the lateral shoot branches emanating from the inoculated branch. Trees were monitored for periods of up to six months for the appearance of GWD.

The relative movement of *N. psidii* in guava xylem tissue was further investigated by stimulating basal shoots to sprout in two to three year old clonal guava plants by girdling the main trunk just above the soil line and laying the plants on their side. After approximately six to eight weeks, the plants were adjusted back to their original vertical position and the two most vigorous newly emerged shoots from the base were selected and the rest were pruned out. One

sucker was eventually selected on each plant and the main stem inoculated at its mid-point by the bark flap method described earlier. Trees were monitored over several months for the appearance of GWD, taking specific note of the basipetal and acropetal movement of the pathogen.

In addition, an experiment was conducted with three specific aims: to observe and record the movement of the disease into the induced basal shoot from the main stem; to observe and record the movement of the disease from the inoculated to the uninoculated partner tree; to determine the rapidity of fungal migration and disease spread in the guava.

Three year old containerized guava plants (cv. Fan Retief) that were induced to produce basal shoots were placed in temperature controlled greenhouses ($28^{O}C$) under ambient light conditions, in pairs (4 pairs in total), approximately 0.5 m apart. Trees were placed under drip irrigation utilizing a single 1L.hr^{-1} dripper per tree (Netafim, Kraaifontein, RSA) and ensuring that drainage water flowed away from the base in closed drains such a way that it did not contact any other pot and flowed outside the greenhouses. In each pair of plants, the main stem of one of the trees in the pair was inoculated as described above and left unpruned for the duration of the experiment. The non-inoculated trees of each pair were pruned at the onset of GWD symptomology in the inoculated paired tree.

Symptoms were recorded on these trees over several months until all the plants had succumbed to GWD. Tissue samples were taken as described earlier from various symptomatic plant tissues over the course of these experiments to determine the presence or absence of *N. psidii* in these tissues. In addition, conidia taken from bark sporodochia that developed on infected trees were tested to determine conidial viability over the course of two years. Conidia from

bark sporodochia were collected and plated out on agar media as described in Chapter 2. Conidia were collected monthly from sporodochia on the same greenhouse trees over the course of two years.

7.2.2 Histopathology

GWD infected field trees from Orchard 2, with GWD ratings of between 1 and 4 were cut back to the scaffold branches and removed from the soil. Trees were sectioned at 300mm intervals with a chainsaw, from the main lateral roots to the main scaffold branches. The chainsaw was surface sterilized by spraying with 70% ethanol between sections. From these large tree tissue sections, a thin slice was removed from the sample by transversely cutting through the middle of the sample. From one end of the two exposed sections, a 10 mm disc was excised by again cutting transversely across the wood grain. Each section sample was placed in a sealed sterile plastic bag and placed in a cooler box containing ice. Within 12 hours, these field sections were aseptically pared and prepared for isolation of *N. psidii,* as described in Chapter 2. A minimum of ten tissue sections were plated out onto each agar plate and this was replicated three times from each tree section. A minimum of three sections of the secondary roots, main roots, main branches and secondary branches were collected from each tree. One main trunk section was extracted per tree. Five trees were used per rating. Ten 125mm^3 wood pieces, taken from across the final tissue section, were surface sterilized and plated onto half strength PDA and incubated at 30°C, as outlined previously (Chapter Two). The extent of vascular tissue colonization by *N. psidii* in trees of different GWD ratings was assessed. Additionally, 4 year old field trees (Orchard 3, Chapter 6, Table 6.1) were inoculated in their secondary roots and branches as per

the key in Figure 9.5. Each treatment comprised of five single tree replicates and each tree was inoculated once as described above. Trees were monitored weekly from the onset of symptoms (approximately 45 days after inoculation) and as trees died, they were removed from the soil, washed by a high pressure water sprayer and sectioned as above and indicated as in Figure 7.5. The extent of vascular colonization by *N. psidii* at approximately the time of death was assessed through microbiological plating of tree tissue sections onto modified WAA (acidified water agar amended with 250mg L^{-1} of ampicillin) agar plates as described earlier.

7.2.3   Secondary branch vs tertiary branch inoculation

Four large concrete containers (1 m diameter and 1.3 m deep) were filled to a depth of 0.5 m with fumigated field soil (clay loam alluvial soil typical of the Nelspruit area). Two year old clonal guava trees (cv. Fan Retief) were established in these containers under an irrigation and fertility regime as described by Du Plessis and Koen, (1984); Koen, (1989); Koen and Hobbs, (1990) and Koen *et al*., (1990). When the trees reached an age of 3 years (November the following year), two trees were inoculated in their secondary branches and two trees inoculated into their tertiary branches (Fig. 9.7) by spraying artificial wounds with a conidial suspension of *N. psidii* (5.4 x 10^{-5} conidia ml^{-1}) and covering the wound with Parafilm[®]. Three wounds were made per tree. The plants were observed for the development of GWD over the course of nine months. This experiment was repeated in winter (June) by removing all the soil and root debris from the containers and surface sterilizing the inner concrete lining with absolute alcohol and igniting it. New soil, fumigated as described earlier, was introduced into the concrete containers and replanted with 2 year old clonal guava plants (cv. Fan Retief). Plants were

196

inoculated as previously described.

7.2.4 Primary root and secondary root inoculations

Five cubic meters of a field soil (clay loam alluvial soil typical of the Nelspruit area) was collected from an uncultivated field and spread across a 50 uM low permeability plastic sheet no deeper than 0.4 meters. The soil was covered with a plastic tarp, the specification of which were the same as the underlying plastic. The edges were sealed with soil, and methyl bromide (75g. M^{-3}) administered by the hot gas method, as described by Willers and Grech (1986a, 1986b). Clonal guava trees (cv. Fan Retief) were established in 6 large concrete containers (1 m diameter and 1.3 m deep) under an irrigation and fertility regime, as described by Du Plessis and Koen, (1984). When the trees reached an age of 3 years, 3 trees were inoculated in their primary roots and 3 trees were inoculated in their secondary roots (Fig. 7.7). Trees with an even number designation were inoculated in the primary root, whereas trees with an odd number designation were inoculated in the secondary roots. The inoculation procedure was as described earlier in Chapter 2 and utilized a conidial suspension of *N. psidii* (5.4 x 10^{-5} conidia ml^{-1}) sprayed onto the cut end of a root and the inoculation site covered with Parafilm® and re-buried.

7.2.5 Field infection studies

Field inoculation of various guava tissues and rhizosphere soil of guava trees (cv. Fan Retief) with *N. psidii*, were carried out at several sites [Orchards 3 and 4 (Chapter 6, Table 6.1)], as well as at the ITSC Friedenheim Experimental Station (near Nelspruit), which at the time of

these experiments was free of GWD. All trees used in these studies were mature (18-27 years) except for those in Orchard 3, where, at the time of these experiments, the trees were 4 years old. Plants were inoculated in the same manner as in the greenhouse studies. Detailed tissue inoculations were carried out primarily at the Friedenheim experimental station due to the lack of GWD. Treatments were as described in Table 7.6. All inoculations involved wounding the targeted tissues except in the unwounded rhizosphere inoculations.

Fruit pedicles were inoculated in two ways: the first was by removing the fruit from the pedicle and pressing the wounded end of the pedicle onto an actively sporulating culture of *N. psidii* (Isolate CSF221), as shown in Figures 7.8 and 7.9. In the second method the fruit pedicle was removed from the fruiting branch and the inoculum were applied to the wounded surface of the branch. Fruit pedicle inoculations were repeated. Lateral roots were inoculated as above, by spraying the cut end of one with a conidial suspension, covering it with Parafilm®, re-burying them and marking the position with a stake. Irrespective of the orchard location or the inoculation site, five inoculations were made per tree, five trees being treated in total per treatment. Control trees were uninoculated. Rhizosphere inoculations were carried out at the Friedenheim Experimental Station as well as in Orchard 3 (Chapter 6, Table 6.1).

Soil rhizosphere inoculations were conducted by producing *N. psidii* inocula by growing the fungus on sterilized wheat seed (50%), guava wood chips (10%), V8 juice [20% (Campbell Soup Company, N.J., USA)] and water (20%). All constituents were thoroughly mixed in a cement mixer and 10 liters of the mix, placed inside autoclavable plastic bags. The media were sterilized, cooled and inoculated with *N. psidii* (CSF221). These bags were agitated by hand every three days, incubated at 30°C under continuous light for two weeks (as described

previously), after which time profuse sporulation had occurred.

At the experimental sites designated for rhizosphere inoculations (Orchards 3 and 4), disease-free guava trees were selected at least 100 meters from any visible GWD infected trees. GWD was present at a very low incidence in Orchard 3 when these experiments were established. At both sites, trees scheduled for treatment each received one bag of the inoculum, which was spread under the tree canopy evenly with a spade and covered with soil, followed by a light irrigation. A further set of trees received the same treatment except root damage was inflicted by stabbing a shovel several times into the rhizosphere underneath the canopy of treated trees to inflict indiscriminate root damage to an approximate soil depth of 40 cm. Control trees were treated with uninoculated media and covered with soil. All trees in these experiments were monitored for the development of GWD over a period of one year. The Controls as well as the treated trees did not receive any other form of treatment except irrigation and fertilization, for the duration of the study. At tree death tissue isolations were undertaken to confirm the cause as GWD. Trees were not pruned for the duration of the trial.

7.3   DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, means were separated using the Waller-Duncan k-ratio t test (Little and Hills 1990).

7.4   RESULTS

7.4.1   Greenhouse Infection Studies

Wounding of plant tissues exacerbated the development of GWD (Table 7.1 and Fig. 7.1). With the exception of leaf and leaf pedicle inoculations, all inoculations preceded by wounding on

all tested tissues were successful in inducing GWD in test plants.

Table 7.1. The influence of wounding and the wounding site on GWD development in greenhouse grown plants

Inoculation site	Percentage of plants with GWD[x]				
	2mo	3mo	4mo	5mo	6mo
Leaf midrib-W	0 a	0 a	0 a	0 a	0 a
Leaf midrib-UW	0 a	0 a	0 a	0 a	0 a
Leaf pedicle-W	0 a	0 a	0 a	0 a	0 a
Leaf pedicle-UW	0 a	0 a	0 a	0 a	0 a
Twig-W	0 a	13b	47 de	73 c	100d
Twig-UW	0 a	0 a	0 a	0 a	0 a
Scaffold branches W	0 a	40d	53 e	100d	100d
Scaffold branches UW	0 a	0 a	0 a	0 a	0 a
Trunk-W	7 b	94e	100f	100d	100d
Trunk-UW	0 a	0 a	0 a	0 a	0 a
1^0 roots-W	0 a	27c	53 e	100d	100d
1^0 roots-UW	0 a	0 a	0 a	0 a	15 b
2^0 roots-W	0 a	0 a	7 b	27 b	60 c
2^0 roots-UW	0 a	0 a	0 a	0 a	0 a
Root suckers-W	0 a	0 a	27 c	27 b	60 c
Root suckers-UW	0 a	0 a	0 a	0 a	0 a

[x] Diseased or dead plants were scored as positive for GWD. 15 single tree replicates per inoculation. Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). W = wounded, UW = unwounded.
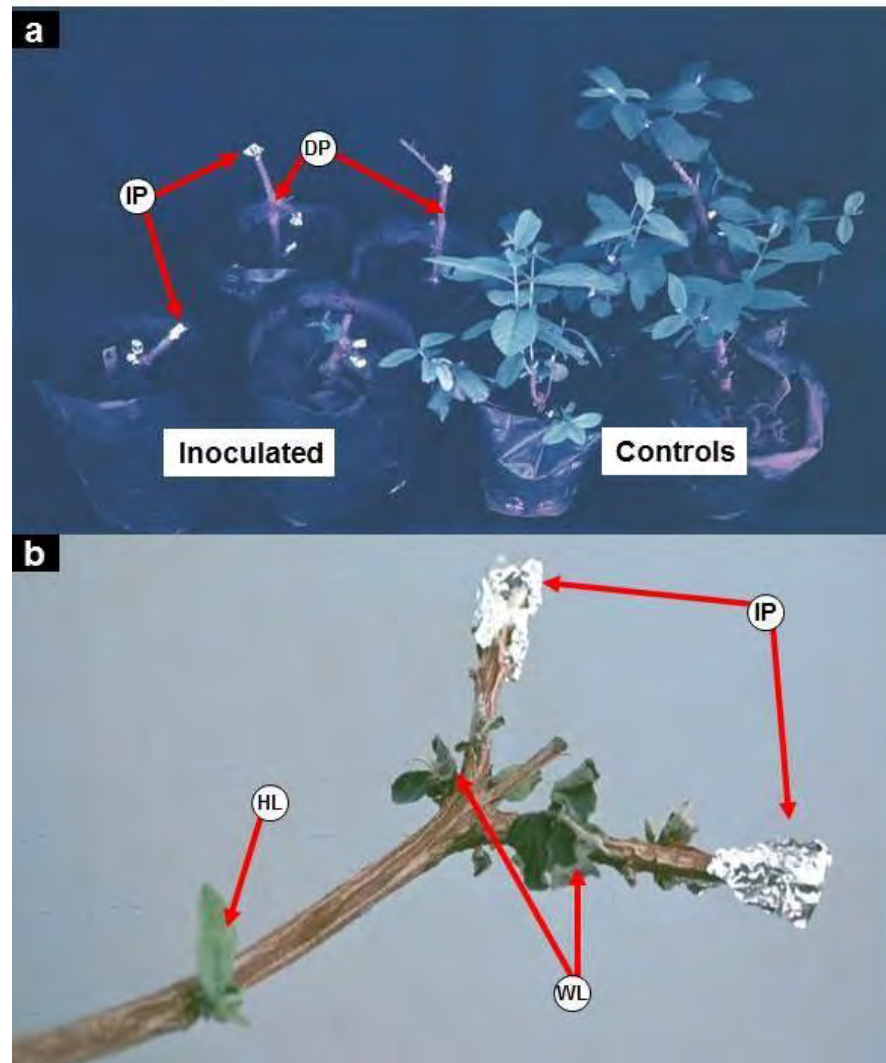
Fig. 7.1. Terminal stem inoculation of 1 year old greenhouse guava plants:
a, dead and diseased plants (DP) previously inoculated with *N. psidii* (left) compared to uninoculated plants (right); b, close up of two lateral stems that were terminally inoculated with *N. psidii* and are exhibiting dieback from the apices.
Note the wilted leaves (WL) close to the inoculation point (IP) as compared to the unaffected healthy leaves (HL) further down the stem.

Primary root inoculations without wounding produced GWD in the test plants but at significantly lower levels than when these plant roots were wounded. Leaf inoculations produced two types of host response following infection (Table 7.2 and Fig. 7.2).

Table 7.2. Mean leaf lesion diameter and percentage mean leaf abscission following leaf mid-rib inoculations with *N. psidii*

	Days after inoculation					
	0	1	3	6	9	12
Mean leaf lesion diameter (mm)[x]	0a	7b	10b	18c	22c	37d
Mean perc-entage leaf abcission[x]	0a	0a	23b	40c	46c	75d
Mean percentage *N. psidii* recovery from symptomatic abscised leaves	0a	0a	85b	93b	91b	94b

[x] Ten leaves were inoculated per plant and the progression of necrosis measured along the midrib axis. Fifteen replicate plants in total. Means followed by the same letter in each row do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).

Three days after inoculation, leaf abscission occurred widely throughout the treatment trees. However, some of the inoculated leaves did not abscise even though large lesions developed on them (Table 7.2 and Fig. 7.2).
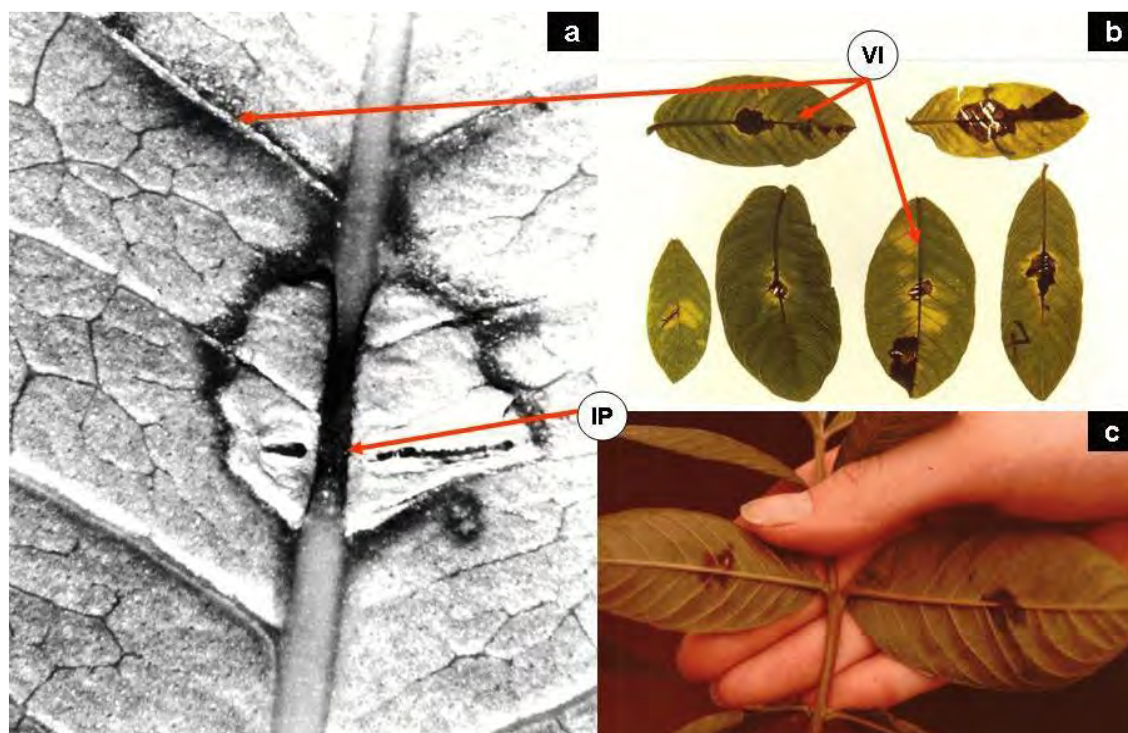
Fig. 7.2. Guava leaf (cv. Fan Retief) inoculations with *N. psidii*:
a, close up of the leaf mid rib inoculation point (IP) showing advancing acropetal vascular necrosis and slight basipetal vascular necrosis; b, multiple examples of leaf vascular infection following inoculation; c, leaf necrosis on localized around the infection point on *in situ* greenhouse plants.

The movement of the *N. psidii* in greenhouse grown guava woody tissues was found to be more rapid acropetally than basipetally (Table 7.3 and Fig. 7.3). In stem inoculated plants that were culturally manipulated to produce basal shoots, basipetal movement of the pathogen was much slower than apical movement (Fig. 7.3) and consistent with the results obtained previously (Chapter 2, Fig. 2.3).

Table 7.3. Progression of *N. psidii* in guava tissues following terminal branch and lateral root termini inoculations

Mean movement of *N. psidii* from the inoculation site (mm)[x].

Inoculation site	Time after inoculation (days).						
	0	14	28	42	56	70	84
Terminal branch (downward progression)	0a	0a	12a	32b	51c	62cd	78d
Lateral root termini (upward progression)	0a	18a	35b	52bc	126d	169e	233f

[x] Movement of the fungus from the terminal inoculation point towards the center of the plant. Means followed by the same letter in each row do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Figures represent the mean of two inoculations per plant. Fifteen plants per treatment.
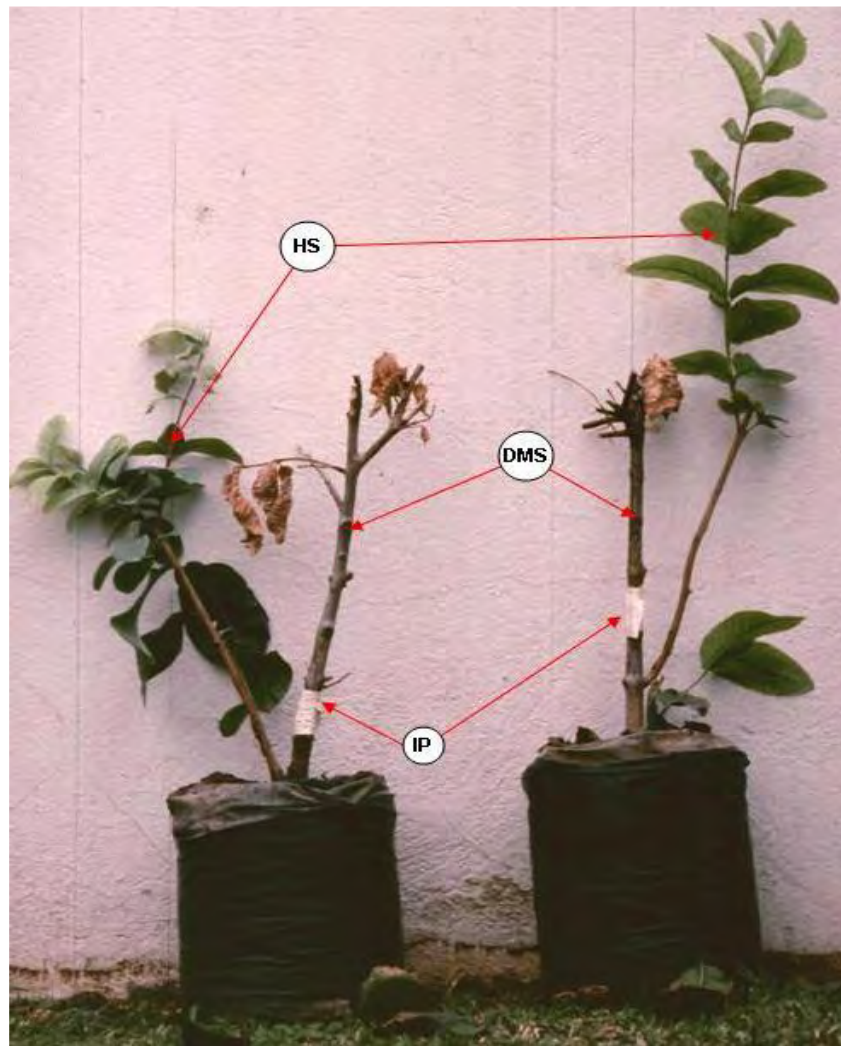
Fig. 7.3. The effect of inoculation of the main stem of one year old clonal (cv. Fan Retief) plants with *N. psidii* on basipetal vascular colonization of lower shoots.
HS = healthy basal shoot; DMS = dead main stem; IP = inoculation point.
Note the survival of the basal shoots.

Downward progression of the pathogen within the scaffold and main stem tissues were also observed to exhibit greater sectoriality than when the pathogen was observed moving upwards in tissues. Examination of the stem sections of pruning wound inoculated plants clearly indicated the restricted xylem and phloem distribution of *N. psidii* (Fig. 7.4) as compared to upward colonization patterns (Chapter 2, Fig. 2.1).
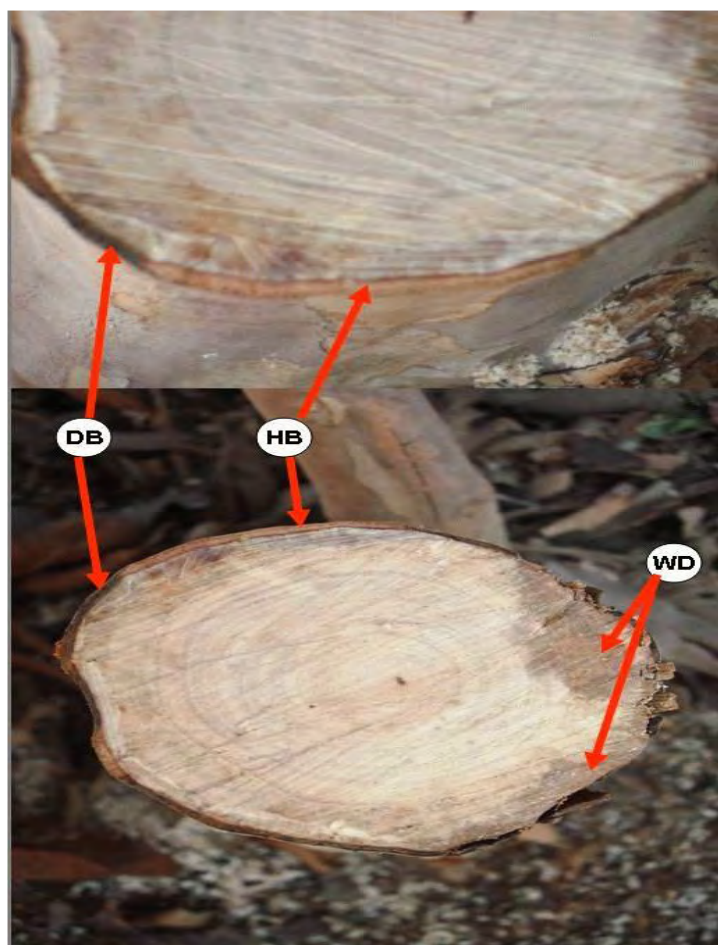
Fig. 7.4. A transverse section through the main trunk of a diseased clonal guava tree naturally infected with *N. psidii* through a pruning wound.
DB = dead bark; HB = healthy bark; WD = wood discoloration.
Note the sectorial wood discoloration in active xylem tissues.

In the paired plant experiments where one of the plants of the pair was trunk inoculated and the other uninoculated, the inoculated trees started to exhibit symptoms within 48 days (Table 7.4).

Table 7.4. Chronology of GWD symptom expression and plant mortality in paired greenhouse plants following main trunk inoculation of one of the tree pair

	Mean period (days)[x] to first symptoms of GWD.	Mean period (days)[x] to complete tissue death.
Inoculated Trees	*	**
Main Trunk	48 a	132a
Basal Shoots	149b	167b
Uninoculated Paired Trees		
Main Trunk	278a	350a
Basal Shoot	315a	368a

[x] Days were rounded up. Figures represent the mean of 4 replicate pairs. The uninoculated tree in each pair was pruned every three months from the onset of GWD symptoms in the inoculated tree. Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (* P=0.01, ** P=0.05).

After 132 days all the inoculated trees exhibited complete aerial canopy collapse of the main trunk with abundant eruptive bark sporodochia evident on the lower trunk in which the conidia persisted in a viable state for least a further two years (data not shown), following the death of the main trunk. Wood discoloration was evident in these collapsed tissues and extended into the canopy. *N. psidii* was isolated from the discolored areas. The basal shoots wilted and died 35 days after the main trunk collapse (Fig. 7.3). Symptoms including apical and sub-apical leaf wilt and leaf drop preceded wilting and plant death (Chapter 6, Figs. 6.6 and 6.7). Wood discoloration (Fig. 7.4) was not evident in these apical and sub-apical tissues at the time of

wilting. *N. psidii* could not be isolated from the areas on the stems that exhibited apical and sub-apical leaf drop. Uninoculated companion trees that were pruned every three months exhibited GWD symptoms on both the main trunk and basal shoots within similar time periods (278 vs 315 days) after the companion tree inoculation (Chapter 6, Figs. 6.3 and 6.5 and Table 7.5). Similarly, total collapse and death of the main trunk and basal shoot of uninoculated paired trees also occurred within similar time frames (350 vs 368 days) from the onset of GWD symptoms in the inoculated companion tree (Chapter 6, Figs. 6.3, 6.6, 6.7 and Table 7.5). The GWD symptom expression in the basal shoot foliage did not express as a unilateral wilt, but rather sectorial wilts at the apical and sub-apical regions of the tertiary branches. *N. psidii* was consistently isolated from symptomatic tissues taken from uninoculated paired companion trees.

7.4.2 Histopathology

The extent of vascular colonization by *N. psidii* was directly proportional to the disease rating stage (Table 7.5). In mature orchards, the pathogen was primarily limited to the roots and trunk. However as the disease progressed, colonization of the secondary branches became more widespread. The effect of secondary root inoculation vs secondary branch inoculation on vascular distribution, indicated that secondary root inoculation resulted in more extensive colonization of plant tissues and more rapid plant death than in the secondary branch inoculations than aerial inoculation (Fig. 7.5).

At the early stages of GWD, *N. psidii* colonization was generally limited to the roots and main

trunk. However as the disease progressed, significantly more colonization occurred in the

secondary branches (Table 7.5).

Table 7.5. Colonization of guava tissues by *N. psidii* in field trees at various disease rating categories

Percentage recovery of *N. psidii* from guava tissues[x]

Disease Rating	Tissue type				
	SB	MB	MT	PR	SR
1	0a	9a	44a	22a	16a
2	12b	44b	100b	32a	24a
3	84c	100c	100b	68b	40b
4	100c	100c	100b	100c	76c

[x] Five trees in each disease category were sectioned. Figures represent the % recovery of *N. psidii* from tissue sections. SR = secondary roots; PR = main roots; MT = main trunk; MB = main branch; SB = secondary branch. Means followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).
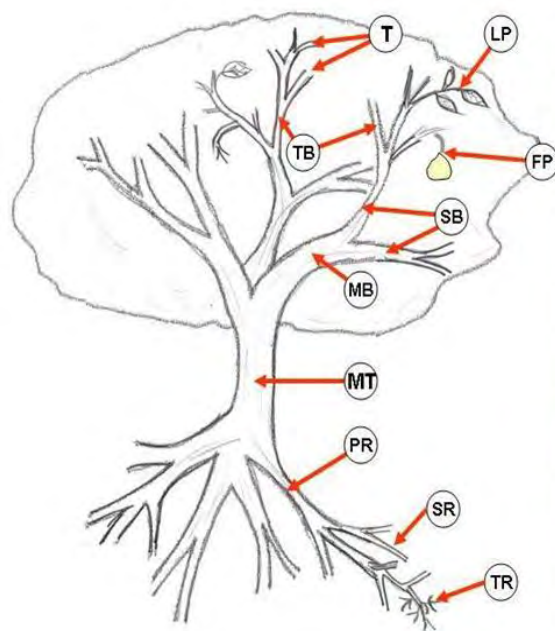
Tree section key
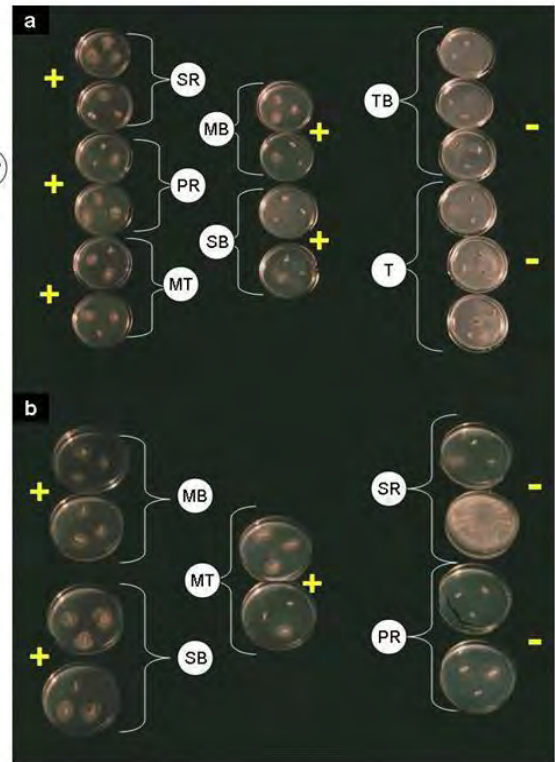
Fig. 7.5. Comparison of *N. psidii* inoculation of secondary branches to inoculation of secondary roots on the rapidity and extent of pathogen tissue colonization in containerized 3 year old clonal (cv. Fan Retief) guava trees at the approximate time of death:

a, secondary root inoculation resulted in tree death at approximately 45-60 days; b, secondary branch inoculation resulted in tree death at approximately 125-165 days.

TR = tertiary roots; SR = secondary roots; PR = main roots; MT = main trunk; MB = main branch; SB = secondary branch; TB = tertiary branch; T = twigs; LP = leaf pedicel; FP = fruit pedicel. + = positive for *N. psidi*i, - = negative for *N. psidii*.

Five replicates per treatment.

7.4.3   Secondary branch vs tertiary branch inoculation

Guava plants inoculated in the summer with *N. psidii* in the secondary branches succumbed to

GWD between 53 and 90 after infection whereas plants inoculated in winter died between 185 and 238 days post-inoculation (Fig. 7.6). *N. psidii* inoculations in the summer of tertiary branches induced tree mortality between 88-158 days, whereas in winter tertiary branch inoculations this range was 195-225 days post inoculation (Fig. 7.6).
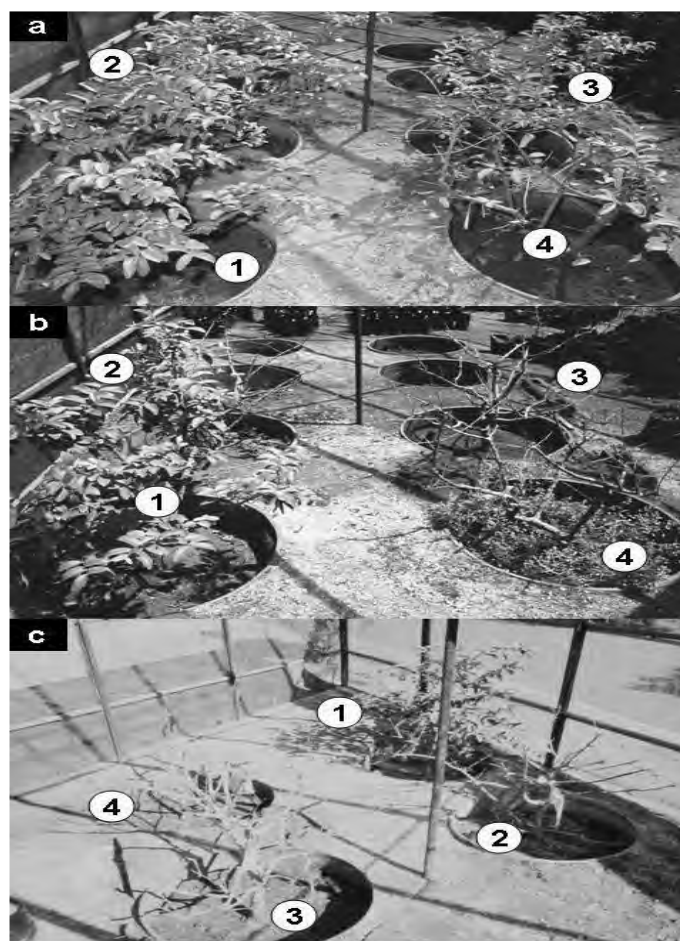
Fig. 7.6. Comparison of *N. psidii* summer inoculation of secondary and tertiary branches in containerized 3 year old clonal (cv. Fan Retief) guava trees:
a, 60 days post inoculation; b, 100 days post inoculation; c, 200 days post inoculation. 1 and 2 = tertiary branch inoculation; 3 and 4 = secondary branch.
Note the higher mortality rate in secondary branch inoculated trees.

7.4.4 Primary root vs secondary root inoculation

Guava plants inoculated in their primary or secondary root system in the summer with *N. psidii*,

did not show any differences in the rate at which they succumbed to GWD (Fig. 7.7). All plants

were dead within 85 days from being inoculated.

Fig. 7.7. Comparison of *N. psidii* inoculation of secondary roots to inoculation of primary roots
on the rapidity of development of GWD in containerized 3 year old clonal (cv. Fan Retief)
guava trees:
a, t = 0 (healthy); b, t = 55 days (symptomatic), c, t = 85 (all dead).
Note that there is no difference in the rate of plant death. Containers 1, 3 and 5 = primary root
inoculated, containers 2, 4 and 6 secondary root inoculated. Container 1 is in the foreground.

7.4.5 Field infection studies

With the exception of the leaf and apical fruit pedicel inoculations, all other inoculation

methods eventually resulted in expression of GWD (Fig. 7.8).

Fig. 7.8. Canopy inoculation of field guava trees:
a, a typical guava leafy branch selected for inoculation; b, *N. psidii* agar culture inoculation of the leaf pedicel abscission point on the branch; c, acropetally advancing leaf defoliation (LD) on a guava branch inoculated with *N. psidii* at a basal leaf pedicel inoculation point (IP); d, GWD symptom expression on a branch previously inoculated at the exposed and wounded fruit (immature) abscission layer, near the branch terminus.

Statistically, inoculation of the primary roots, main trunk and or scaffold branches resulted in the fastest onset of GWD symptoms after inoculation (Table 7.6).

Table 7.6. The influence of the wounding site on the field development of GWD

Inoculation site	Elapsed time period from *N. psidii* inoculation until GWD development (percentage of trees exhibiting symptoms)[x].				
	1mo	3mo	6mo	9mo	12mo
Leaf pedicel	0 a	0 a	0 a	0 a	0 a
Fruit pedicel[a]	0 a	0 a	0 a	0 a	0 a
Fruit pedicel[b]	0 a	0 a	0 a	20 b	20 b
Twig	0 a	30b	40 d	80 d	100d
Scaffold branch	30b	80c	100e	100e	100d
Trunk	40c	80c	100e	100e	100d
Rhizosphere	0 a	30b	30 c	80 d	100d
Rhizosphere (wounded)	0 a	30b	60 d	100e	100d
Root Suckers	0 a	0 a	20 b	40 c	60 c
Primary Root	0 a	30b	100e	100e	100d

[x] Diseased or dead plants were scored as positive for GWD. Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). 10 single tree replicates per treatment. [a] The fruit pedicel was inoculated at the apical (fruit) end. [b] The fruit pedicel was inoculated at the branch end.

Interestingly, fruit pedicel inoculation (Fig. 7.9) at the fruit end did not produce GWD symptoms over the 12 month observation period.

Fig. 7.9. Canopy inoculation of field guava trees:
a, inoculation of the fruit pedicel abscission wound by direct contact with a sporulating PDA petri plate agar culture of *N. psidii*; b, inoculation of the fruit pedicel abscission wound with *N. psidii* by utilizing agar bocks from an actively growing PDA culture; c, wound inoculation of a branch terminus with a spore suspension of *N. psidii*; d, wound inoculation of a branch terminus utilizing agar bocks from an actively growing culture of *N. psidii*.

Fruit pedicel inoculation at the branch end did result in low levels of GWD expression (Table 7.6), but this result could not be repeated in subsequent experiments (not reported here). Rhizosphere inoculations with or without root wounding were not significantly different from one another at the Friedenheim test site (Table 7.6) over most of the assessment periods. At the 12 month assessment all plants in both treatments died from GWD (Table 7.6).

7.5 DISCUSSION

These studies confirmed the earlier findings of Chapter 2, in which wounding was found to be necessary for *N. psidii* to infect guavas. With the exceptions of leaf and fruit pedicel inoculations (fruit terminus), all guava plants that were wound inoculated with *N. psidii* succumbed to GWD (Tables 7.1, 7.6, and Fig. 7.1). In both greenhouse and field trials, trunk inoculation resulted in the most rapid onset of GWD and plant death. Interestingly, inoculation of the branch terminus of the fruit pedicel in field inoculations, resulted in the development of GWD (albeit at low levels) in contrast to the fruit terminus inoculations which did not develop GWD (Table 7.6), as discussed more fully later in this section.

Inoculations of unwounded primary roots did result in one replicate becoming infected with GWD at the six month assessment period, but at a significantly lower level than the wounded inoculation treatment (Table 7.1). This finding may be the result of biotic root wounding factors (animal and/or human cultivation practices) and/or abiotic factors (such as soil cracking). Inoculations of the main trunk resulted in the most rapid development of GWD and subsequent tree death.

Leaf inoculations resulted in the development of a lesion (Fig. 7.2) which again moved more rapidly acropetally than basipetally. The fungus did not breach the leaf pedicel and in most cases the leaf abscised if the lesion progressed close to the leaf base. Leaf inoculation did not result in the systemic colonization of the plant by *N. psidii* or plant death in any of the multiple leaf inoculation studies under controlled or field conditions. Inoculated leaves showed the

highest abscission after three days, typically with leaf lesion areas covering approximately a third of the leaf (Fig. 7.2). The lesion diameter generally exhibited a linear increase with time (Table 7.2). *N. psidii* was isolated in high frequencies from inoculated leaves (94%).This is the only known guava tissue that exhibits this hypersensitive response to *N. psidii* and in so doing averts plant death. Leaf inoculation may be a useful prescreening tool in the selection of resistance to *N. psidii* and/or the utilization of this technique to assess new strains of the pathogen, as this technique was found not to induce overall tree death, the same plant specimens can be used repeatedly. Pathogenicity of *N. psidii* to several types of tissue in the greenhouse and field, clearly showed that this specialized plant pathogen is most aggressive as a colonizer of xylem tissues of the main roots and main trunk (Tables 7.1 and 7.6). Infection of the leaf or fruit pedicel was not observed naturally over the course of these studies and these infections routes are not believed to be significant in field epidemics based on the results of these experiments. However, it is important to note the ability of *N. psidii* to infect a very diverse range of guava tissues, and as such, any phytosanitary measures adopted to ensure disease free plants from nursery stock should take cognizance of these findings.

The progression of *N. psidii* in the vascular system of guava was found to be faster acropetally than basipetally (Tables 7.3, 7.5 and Fig. 7.3), which is typical of many fungal vascular wilting pathogens (Mace *et al.*, 1981). In these experiments the pathogen migrated acropetally at approximately 2.8 mm.day^{-1} as compared to its basipetal rate of approximately 0.9 mm.day^{-1}. This represents a three-fold difference in the acropetal versus basipetal xylem colonization rate. This is of particular importance in terms of the management of aerial infection of pruning wounds. Transverse distribution patterns in the main trunk xylem and phloem tissues of

basipetally migrating *N. psidii* were more restricted (Fig. 7.4) than for acropetal migration (Chapter 2, Fig. 2.1). The downward movement of the pathogen in woody tissues was generally restricted to one sector of the stem and seemed to colonize transversely primarily via the bark. This may be indicative of xylem vessels impairing the basipetal movement of mycelia due to the formation of tyloses in the xylem vessels (Chapter 3, Fig. 3.6). Most vascular diseases (particularly fungi) of plants colonize the comparatively nutrient poor xylem vessels compared to the carbohydrate rich phloem. This may be partially due to the fact that the xylem is largely composed of dead conductive cell (tracheids) under low osmotic pressure as compared to the phloem that is living, possesses sieve plates and is under much higher osmotic pressure and hence more difficult to colonize by fungi (Yadeta and Thomma 2013). Earlier in these studies (Chapter 3), *N. psidii* was observed breaching xylem pits, which have diameters of approximately 2-3 μm. However the pathogen has not been observed navigating phloem sieve tube pores and this may be due to their small diameter [(+/- 0.5 μm), Brussieres, 2014].

Protection of pruning wounds and the augmentation of the host response to prevent infection of pruning wounds, if possible, may be useful in reducing the infection progression of *N. psidii* utilizing this mode of tissue entry. Chemical protection of pruning wounds to prevent fungal infection is widely used in commercial tree and vine cultivation for several maladies (Rolshansen *et al.,* 2010). In addition, cultivation equipment including pruning shears can be a repository for infective propagules of GWD (Grech, 1983, 1987) as well as with other diseases (Agusti-Brisach *et al*., 2015).

In the paired greenhouse inoculation experiments [where the plants were not physically

connected; nor had any connection through irrigation delivery or drainage, (Chapter 6, Figs. 6.3, 6.5 and 6.6], the inoculated trees became symptomatic for GWD on average at 48 days (Table 7.4). This was followed by a rapid collapse, at on average 132 days, of the aerial canopy supported by the main trunk. These tissues exhibited wood discoloration and *N. psidii* could be isolated from these areas on the stems. The tree crown and subterranean tissues remained unaffected up until 149 days post-inoculation, as evidenced by the lack of GWD symptoms in the basal shoots (Table 7.4). An example of basal shoots surviving after main trunk of collapse is seen in Figure 7.3. Although the basal shoots did survive for a significantly longer period than the main trunk, they did eventually succumb to GWD with a mean period of 167 days post main trunk inoculation (Chapter 6, Fig. 6.6). This reinforced the earlier findings in this study of a three times greater acropetal velocity in vascular tissue compared to the basipetal velocity (Table 7.3) of *N. psidii*. The progression of canopy symptoms in the basal shoots of the trunk inoculated trees were sectorial and non-uniform (Chapter 6, Figs. 6.6 and 6.7). Leaf and branch wilting was observed in apical and sub-apical regions of the canopy tissue. These basal shoot GWD symptoms were markedly different from those observed in the aerial portions of the main trunk inoculated trees, where a unilateral canopy wilt occurred. Basal shoots exhibited both apical and sub-apical wilting of tertiary branches prior to overall collapse of the canopy (Chapter 6, Figs. 6.6 and 6.7). Tissue isolations from these sectorially wilted shoots (Chapter 6, Fig. 6.10) did not indicate the presence of *N. psidii*, and as such, it was postulated that this observed sectorial and sub apical wilting was associated with a putative systemic plant wilting factor. Plant wilting toxins that have been reported previously in a wide variety plant vascular wilting disease complexes (Stoddart and Carr, 1966; Van Alfen and Turner 1975, van Alfen and McMillan 1981, Van Alfen 1989 and Graniti*.,* 1991). These observations led to an

investigation (Grech, unpublished) of the role of plant wilting toxins in GWD (not presented herein) and the results from these studies are discussed later in this thesis. These symptoms are often observed in field trees and may have been previously mistaken in their entirety for aerial infection courts (Hough and Kellerman 1986).

Basal shoots emanating from the inoculated trees became symptomatic later and survived significantly longer than the main trunks (Table 7.4). This is in contrast to the uninoculated companion trees of each replicate pair, where there were no differences between the basal shoot and main trunk in terms of when they became symptomatic and how long they survived (Table 7.4). This would indicate that infection of the basal shoots and the main trunk occurred in a similar temporal period and probably through the same infection courts based on earlier finding as to the progression of *N. psidii* in different guava tissues (Table 7.1). The uninoculated paired companion trees became symptomatic for GWD in both the basal shoot canopy and the main trunk canopy (Chapter 6, Fig. 6.5) at multiple locations. These tissues exhibited wood discoloration from the pruning wood moving basipetally and *N. psidii* could be isolated from these areas on the stems. This would indicate aerial infection of the pruning wounds from inocula emanating from the inoculated paired tree (Chapter 6, Fig. 6.5). The statistically similar interval from inoculation to symptom expression to plant death in the paired tree basal shoots and main trunk, indicated not only similar infection courts but also slower basipetal fungal tissue progression. This would therefore explain why these companion trees took a significantly longer time from symptom expression to death than in the main trunk inoculated trees (Table 7.4). Erumpent sporodochia on inoculated, paired trees were considered the putative source of conidial inoculum. These structures were found to contain conidia that in these studies

remained viable for at least two years following tree death. This may not be the case in the field where conidia are easily dislodged from the erumpent, exposed sporodochia due to weathering erosion. However, previous studies (Stipes and Phipps 1974) have shown sporodochia-derived conidia that developed from woody tissues in diseased trees remained viable in the field for periods of approximately two years.

The extent of vascular colonization of field guava trees was directly proportional to the tree disease rating (Table 7.5). *N. psidii* was found to be most consistently recovered across all disease stages from the main trunk. GWD symptom expression intensity increased as *N. psidii* progression into woody tissues advanced. The extent of colonization of guava tissues by *N. psidii* at the time of tree death, indicated that secondary root inoculations resulted in greater tissue progression than that of secondary branch inoculations (Fig. 7.5).

Containerized three year old guava trees inoculated in summer in their primary or secondary root system succumbed to GWD at similar rates (Fig. 7.7). All plants were dead at eighty five days post inoculation. In contrast to these root studies, rates of mortality in trees inoculated in their secondary or tertiary branches differed in terms of life expectancy. Plant mortality was more rapid in summer inoculated secondary branches (53-90 days) versus tertiary branches [88-158 days (Fig. 9.7)], which was consistent with previous results in this study where acropetal migration of *N. psidii* in guava xylem tissues were found to be more rapid than basipetal migration (Table 7.3). Winter inoculations produced a similar result, in so far as secondary branches resulted in plant death between 185-238 days, whereas in the tertiary branches, this interval was 195-225 days. As these inoculation where conducted in shade

houses at ambient temperatures, these winter inoculations actually resulted in the onset of plant death the following summer/autumn, and as such, *N. psidii* may have remained dormant in the inoculation wounds until favorable fungal growth temperatures occurred.

Inoculations of various tissues of field trees with *N. psidii* produced similar results to earlier greenhouse inoculation studies (Table 7.1). Of interest was the difference between inoculation of the basal end (stem end) or the apical end (fruit end) of the fruit pedicel. The fruit pedicel was found to be receptive to infection by *N. psidii* only at the stem end (Table 7.6) albeit at low levels compared to other tissues inoculated. Guava fruit do not naturally retain a pedicel as certain other fruit do, such as cherry (Webster and Looney 1996), and naturally abscise from the tree at the fruit end of the fruit pedicel (personal observation[2]). Although this type of wound was found to be unreceptive to *N. psidii*, commercial picking of guava fruit in many countries including South Africa occurs before fruit abscission and in some cases (high value fresh market guava varieties such as those found in Asia), fruit secateurs are used (Kwee and Chong, 1990). Fortunately guava harvests occur in winter when *N. psidii* growth, and subsequent GWD development, is much slower (Chapter 3).

Whereas in aerial tissues, wounding was generally a necessary prerequisite for *N. psidii* infection, unwounded rhizosphere inoculations induced GWD in the field [albeit at slightly lower levels compared with the wounded treatment (Table 7.6)]. At 24 months (not reported here), all the tree replicates in the wounded and unwounded tree rhizosphere inoculations were

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[2] Grech, N.M. Field observation of guava fruit development, abscission and commercial harvests in South Africa, Mexico, U.S.A.(Hawaii) and Asia.

dead. It is postulated that natural wounding agents in the soil may result in sufficient wounding for penetration by *N. psidii*. Natural wounding agents in the soil have been implicated in many root invading pathogenic fungi (Bruehl, 1987). Recently in Taiwan, there has been renewed interest in the rhizosphere etiology of GWD (Hong *et al.*, 2013, 2014). Previously root infection was not considered as significant as shoot infection in the transmission of the disease, but these studies contradict those earlier findings. The authors report propose that root to root transmission of *N. psidii* is actually the dominant mode of transmission in guava orchards which is an agreement with these studies.

Systemic colonization of guava tissues occurred with all inoculations [except with the leaf and leaf pedicle (fruit end) inoculations]. Rapid progression of GWD was observed following plant inoculation (Table 7.2, Figs. 7.8 and 7.9). Plant death occurred most rapidly with the trunk, primary root and scaffold branch inoculations resulting in plant death at 6 months.

The pathogen in woody tissues has a substantial substrate base of considerable duration and it was found in these studies to persist in these tissues for up to a year (Grech, unpublished), without significant reductions in the ability to recover it through tissue isolation. The strong parasitic activity of *N. psidii* results in a rapid systemic distribution of the fungus in the host prior to host death. Colonization of large sections of the trunk and roots allows the pathogen to sustain itself for a long period of time. These findings are consistent with the dead tissue longevity reported in some other vascular wilting fungi (Yount, 1955). *N. psidii* was found to possess the ability to produce cellulase in culture. This was further confirmed by the dyed substrate test. Cellulose digestion confers a significant advantage on a xylem colonizing

vascular wilting fungus allowing the fungus to infect, colonize and maintain growth even after host death. Fungi from the genera *Gliocladium*, *Acremonium* and *Penicillium* are well known in their ability to produce cellulases (Barkai-Golan and Karadavid, 1991; Jagpal and Garg, 1995; Kansarn *et.al.* 2000). The discovery that cellulase activity was present in both the liquid growth medium as well as in actively growing cultures, indicated a cell wall degrading capability of *N. psidii* that would be critical in the colonization and infiltration of plant tissues by the fungus, as is the case in other plant vascular wilting complexes (Cooper, *et al.,* 1978). It would seem logical that this fungus possesses significant enzymatic tissue digestion capability beyond that of cellulases alone that would confer significant advantages in the progression of tissue colonization and nutrient acquisition. Previous microscopic studies by the author (Chapter 3, Figs. 3.5, 3.6 and 3.7) indicated that *N. psidii* moved laterally across xylem vessels by degrading and penetrating pits in the xylem cell wall. Earlier histopathological studies (Chapter 3) utilizing light and scanning electron microscopy of wood sections from *N. psidii* infected trees clearly illustrated the progression of the hyphae through the xylem vessels with advancing disease. Electron micrographs revealed the unique adaption of this organism in terms of possessing two hyphal types and their importance in vascular colonization of guava trees. It appears that the narrower hyphal filaments breech and penetrate the xylem wall pits allowing for rapid lateral spread across and through xylem tissues. These pits appear to have amorphous occlusions similar to those described by Ouellette (1978), possibly produced as a response to fungal invasion. The production of endohyphae by *N. psidii* (Chapter 3, Fig. 3.4) may afford protection to the fungus as it migrates in the xylem vessels, as well as auto-recycling

nutrients (Menge, personal communication, 1996[3]). The appearance of vascular occlusions in the xylem vessels of trees infected by *N. psidii* usually were found to occur after extensive host invasion, resulting in the widespread impairment of the transpiration stream. Gelation and tyloses formation was not observed to occur promptly around the infection court (although these vascular occlusions were detected in advance of more established infections of both the root woody stems and roots). The absence of rapid post infection formation of tyloses in the xylem is generally is indicative of a susceptible host-pathogen relationship according to the studies of Bishop (1983) and later by Gaudet and Kokko (1984) as well as Bruehl (1987). Resistant hosts or hosts exposed to non-pathogenic microorganisms, quickly localize them by vascular occlusions (Beckman and Halmos, 1962; Bishop and Cooper, 1984; Gbaja and Chant, 1984 and Bruehl 1987) postulated that resistance of plants to vascular wilting pathogens depends on the early response to the fungus within the xylem vessels.

The discovery from these studies that *N. psidii* can in persist guava woody tissues for periods up to twelve months have important repercussions on field management of GWD (Grech, unpublished). Obviously the on-site destruction (usually by fire) of as much dead plant material as possible is critical, with care not to move material from orchard to orchard, as suggested for other tree wilting diseases (Phipps and Stipes, 1976). Once destroyed, the low soil persistence of *N. psidii* is advantageous, particularly where soil fumigation is applied. Soil fumigation with methyl bromide is effective in destroying pathogens in the soil but also in root pieces

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[3]Professor Emeritus John Menge, Dept. of Plant Pathology, University of California, Riverside, CA 92521, USA.

smaller than 25 mm diameter (personal communication, Mr David Marks[4]). Hence methyl bromide soil fumigation may be effective in eliminating residual *N. psidii* inocula resident in small root pieces left in the soil after tree removal. Agricultural use of methyl bromide is increasingly restricted and as such alternative fumigants such as methyl iodide, which has been shown to be an equally effective soil fumigant (Ohr *et al.*, 1996), would likely also be effective in eliminating *N. psidii* in the soil.

N. psidii is an aggressive, efficient wound pathogen of guava that has evolved a range of mechanisms (such as two spore types and endohyphae) adapted to infect guava tissues both in the aerial canopy and the root system. Transmission of *N. psidii* occurs through air and/or in water (Chapter 3). The reduced basipetal colonization rate compared to the more rapid acropetally xylem colonization rates would suggest why root infections induced plant death more rapidly than aerial infection.

Several techniques developed in these studies warrant further investigations as to their suitability to screen guava germplasm for tolerance to *N. psidii* such as the leaf inoculation technique and the germling radical assay.

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CHAPTER 8: RHIZOSPHERE ETIOLOGY OF GUAVA WILT DISEASE: GREENHOUSE AND FIELD INFECTIVITY BY *NALANTHAMALA PSIDII*; POTENTIAL BIOCONTROL WITH A CLOSELY RELATED FUNGUS, *NALANTHAMALA VERMOESENII*; IMPACT OF ROOT ANASTOMOSIS ON DISEASE SPREAD

ABSTRACT

Rhizosphere inoculation of greenhouse grown clonal guava (cv. Fan Retief) with *Nalanthamala psidii* (Biourge) Schroers, combined with simulated soil tillage, resulted in guava wilt disease (GWD) development within 60 days, followed by plant death within 30-60 days. *Nalanthamala vermoesenii* (Biourge) Thom inoculated into the rhizosphere prior to inoculation with *N. psidii* did not induce any adverse effects on the guava, but failed to protect against infection with *N. psidii*. Inoculation with *N. vermoesenii* alone increased plant dry shoot mass by 58%. Natural root grafting in orchard surveys was commonplace in older orchards (>8 years old), predominantly in the row (+/- 70%), but also between rows (+/- 30%). The extent of root grafting was heavily influenced by orchard cultural practices. Glyphosate was shown to have utility as a tool in quantitatively assessing orchard root anastomosis non-invasively, but the technique requires further refinement. Mechanically restricting or severing root to root contact and/or root anastomosis in containerized or field trees significantly reduced the rate of disease spread in guava (cv Fan Retief) by 78% (over 290 days) and 56% (over 360 days), respectively. Sequential disease transmission between adjacent mature root grafted trees indicated transmission of *N. psidii* via primary and secondary root grafts, and supports previous statistical outcomes from these studies as to the non-random nature of GWD spread in orchards. *N. psidii* ingress from a diseased tree to a healthy tree crown from a root graft, was usually on a singular primary root and at that time, the newly infected tree may be symptomless for GWD. The microbial composition of guava feeder and tertiary roots during the 12 months following plant death from GWD was largely constant with the exception of *Pythium* and *Phytophthora* species, which were undetectable 2 months *postmortem*. A diagrammatic representation of the putative disease cycle of *N. psidii* on guava was developed.

8.1   INTRODUCTION

Prior research (Chapter 5) showed that spread of this pathogen in the field follows a non-random pattern from tree to tree, a characteristic of many vascular wilting fungi (Appel and Maggio, 1984; Elliot, 2010). In the field many factors have been implicated in expediting the development of guava wilt disease (GWD) including pruning, irrigation, root grafting, mechanical weeding and sucker removal and poor sanitation practices. *Nalanthamala psidii* (Biourge) Schroers produces two distinct conidial spore types (Chapter 3), one hydrophobic (Type 1) and the other hydrophilic (Type 2). As such, it is well adapted to dissemination in both air and water, as previously reported by Wu and Hong (2012) and earlier in these studies (Chapters 4 and 5).

The non-random spread of guava wilt in the field may be related to root grafting between trees. Disease spread via root grafts is common in Dutch elm disease (Kendrich and Molnar, 1965; Anonymous, 1993a) and in oak wilt (Appel *et al.,* 1989, 1992). In commercial guava orchards where the trees are clonally produced (and are therefore genetically uniform), it is likely that root grafting is widespread, with self-recognition taking place.

Fungal vascular wilting pathogens such as *Fusarium oxysporum* [Schlecht. (Snyder and Hansen)] have evolved efficient mechanisms for long term survival in the soil, usually through the production of recalcitrant chlamydospores (Booth, 1971). In most cases, soil fumigation used to be the only commercially viable method of reliably removing these pathogens effectively from the soil (Ploetz, 1990, 2007). Unfortunately these treatments do not have long lasting effect, are very costly (Herbert and Marx, 1990), are generally environmentally harmful (Anonymous, 1993b), with the most effective products such as methyl bromide, no longer

being available in the market. *N. psidii* produces chlamydospores, but the soil longevity of this fungus is unknown, as is its longevity in diseased or dead root pieces. Fungal survival in dead root pieces is of major importance in root infecting vascular wilt diseases (Yount, 1955). Replanting of *N. psidii* infested lands immediately after dead tree removal with young trees has almost universally resulted in rapid tree death.

The aims of these studies were to investigate the soil and root etiology of GWD, particularly in terms of the transmission of the pathogen through the rhizosphere and the importance of root grafting in this process. An initial determination of the viability of a closely related fungus, *Nalanthamala vermoesenii* [(Biourge) Schroers]*,* as a potential biocontrol agent on guava roots was investigated.

8.2   MATERIALS AND METHODS

8.2.1   Soil inoculation

Inocula were prepared by placing wheat seed (30 kg) and V8 juice [5 L (Campbell Soup Company, USA)] in a standard 120 L capacity autoclavable plastic bag. A total of four bags were prepared, tumble mixed and autoclaved twice in succession. When cooled, the culture medium contained in the bags was inoculated with *N. psidii* (CSF221). These bags were then placed in an incubator set at 30°C with 12 hour incandescent light illumination cycles. Every three days these bags were removed from the incubator, inspected and the contents tumbled inside the bag to ensure thorough mixing. Additionally *N. psidii* was grown in Czapek dox broth liquid culture (20 L in total of broth culture media) at 30°C under constant agitation with 12 hour incandescent light illumination cycles, as described in Chapter 4. Both sets of inocula

were incubated for 14 days after which the contents of the liquid culture was removed and macerated in a culinary blender (Kenwood, USA) set at 'puree', for approximately 2 minutes. This was then poured into a surface sterilized mixing vessel of a small cement mixer (50 L capacity) in which the inocula grown on wheat seed had been added. The mixture was agitated for 15 minutes and then placed back into the autoclavable plastic bags and refrigerated at 10°C until used (within 6 hours). A small sample was retained to test the purity and viability of the inocula. Similarly a non-pathogenic strain of *N. vermoesenii* (IMI 40231) was grown and prepared as for the *N. psidii* soil inoculations. Ten plants were used per treatment and the experiment was repeated twice.

Containerized guava plants allocated for soil inoculation studies were pruned back to their main scaffold branches and the pruning wounds immediately painted with copper oxychloride (250g. L^{-1}) plus latex paint (250g. L^{-1}) in water. The plants were left for 1 week in this condition before being moved to the greenhouse compartment where soil inoculations were to be conducted.

Soil inoculations with *N. psidii* and *N. vermoesenii* comprised initially of lightly cultivating the soil (to a depth of 10 cm) of the pruned guava plants, followed by several stabs into the soil with a hand garden fork to induce root damage. Additionally a 2 cm diameter soil core was excavated in each plant container to depth of 10 cm, equidistant between the plant stem and the edge of the plant container. Treatment plants each received 10 g of inoculum. This inoculum was spread over the soil surface of the containerized plant and into the soil core. Immediately following this procedure, the plants were watered lightly by hand.

Soil inoculations of *N. vermoesenii* were undertaken to determine if a non-pathogenic strain of *N. vermoesenii* applied prior to *N. psidii* could reduce GWD development. Treatments are as described in Table 8.1. *N. vermoesenii* was applied followed by *N. psidii* 48 hours later (without additional root wounding). Single inoculation treatments all received inocula at the onset of the experiment. Control plants only received the soil cultivation treatment followed by irrigation. New shoot growth (dry mass) was assessed at 90 days and plant mortality was assessed over the entire experimental period of 180 days.

8.2.2   Large containerized plant inoculation studies

The effects of root anastomosis on GWD progression was investigated in large water-porous concrete trays [(3 x 1.5 x 0.3m) Fig. 8.2]. In one of the trays, plastic boards were positioned perpendicularly across the long axis of the trays and spaced every 25 cm to accommodate one tree column of 12 trees. Once installed these plastic barriers would isolate tree columns. The plastic boards were sealed with a silicon sealant along the edges that were in contact with the inner concrete surfaces of the tray. One day after sealing the compartments, they were checked for water tightness. The trays were filled with a standard nursery soil mix and gently compressed with a spade. The soil was lightly irrigated and 24 hours later, clonally propagated 1 year old guava plants (cv. Fan Retief) were planted by hand and bedded into the soil at a 0.25 x 0.125m tree spacing (144 trees per tray). After planting, the soil line was maintained approximately level with the top of the boards. In the second container, guava trees were planted identically but without any barriers between tree columns.

One month after planting, the guava plant occupying the extreme south east corner of each tray, was pruned back to the main trunk, and immediately inoculated with a CMA culture of *N. psidii* as previously described in Chapters 3 and 4). Plants were monitored weekly over approximately 10 months and assessed for the development of GWD. In the event of the onset of GWD symptoms or tree death, trees were cut off at the soil line, the main stem wounds immediately painted with copper oxychloride (250g. L^{-1}) plus latex paint (250g. L^{-1}) in water and the aerial tissues removed and sectioned for isolation of *N. psidii*. Care was taken to remove the above ground canopy with minimal soil disturbance and before sporulation could take place. All plants were pruned back to the main stem, 45 days after initiating the experiment, due to excessive canopy growth exacerbating tree to tree variability. Immediately following pruning, the pruning wounds were painted with copper oxychloride (250g. L^{-1}) plus latex paint (250g. L^{-1}) in water.

At the termination of the experiment, the remaining plants were dug up, washed and examined for root contact and anastomosis.

8.2.3 Field rhizosphere inoculations

Soil rhizosphere inoculations were conducted by producing *N. psidii* inocula by growing the fungus on sterilized wheat seed (50%), guava wood chips (10%), V8 juice [20% (Campbell Soup Company, N.J., USA)] and water (20%). All constituents were thoroughly mixed in a cement mixer and 10 liters of the mix, placed inside autoclavable plastic bags. The media were sterilized, cooled and inoculated with *N. psidii* (CSF221). These bags were incubated at 30º C under continuous light for two weeks (as described previously), after which time profuse sporulation had occurred.

At experimental sites where GWD was present (Orchards 3 and 4), disease-free guava trees were selected at least 100 meters from any visible *N. psidii* infected trees. The pathogen was present at a very low incidence in Orchard 3 when these experiments were established. At both sites, trees scheduled for treatment each received one bag of the inoculum, which was spread under the tree canopy evenly with a spade and covered with soil, followed by a light irrigation. A further set of trees received the same treatment except root damage was inflicted by stabbing a shovel several times into the rhizosphere underneath the canopy of treated trees to inflict indiscriminate root damage. Control trees were treated with uninoculated media and covered with soil. All trees in these experiments were monitored for the development of GWD over a period of one year. The Controls as well as the treated trees did not receive any other form of treatment except irrigation and fertilization, for the duration of the study. At tree death tissue isolations were undertaken to confirm the cause as GWD. Trees were not pruned for the duration of the trial.

8.2.4 Field root contact and anastomosis

To determine the initial incidence of root anastomosis in orchards, glyphosate (Roundup®, 360g.L^{-1} a.i., Monsanto, Bryanston, RSA) herbicide was applied as follows: a 0.15%v/v solution of Round up was prepared with 1% w/v ammonium sulfate added to acidify the solution. This solution was applied by a motorized low drift sprayer at 5 liters per tree to 25% of the tree canopy in the plane of the tree row. Adjacent trees were shielded with a plastic sheet to avoid spray drift. Trees were observed for 3 months to determine whether any herbicidal

symptom of Roundup® was noticeable on the adjacent trees. This was repeated three times in the orchards surveyed for root grafting.

Surveyed orchards were assessed for the incidence of lateral root contact and/or grafting (anastomosis). Older orchards were compared to younger orchards to determine the level of root contact/grafting. Orchards 2 and 3 (Chapter 5, Table 5.1) at 22 and 4 years old, respectively, were selected to investigate the incidence of tree to tree root/contact grafting. A preliminary investigation was made whereby a root observation trench was excavated between trees by gently loosening the soil with a garden fork and washing the soil away using a high pressure vehicle mounted sprayer. Ten such trenches were excavated per orchard and the level of root to root contact/grafting of lateral roots were assessed. In one case where tree to tree *N. psidii* spread had appeared to occur, the diseased tree next to the dead tree was excavated and sectioned and tested for the presence and vascular distribution of *N. psidii* by microbiological assaying for the fungus, as described earlier.

In Orchard 2, 15 groups of three trees were selected that were isolated by at least 100m from *N. psidii* infected trees. Five of the three tree replicates had the center tree trunk inoculated with *N. psidii*. In the second group of five, three tree replicates were similarly inoculated but also had a 0.75 m soil ripping treatment between the trees to sever any potential root contact/grafting. The other five, three tree replicates served as Controls. Pruning was suspended for the duration of the experiment and trees were treated with insecticides to control the SBB. The trees were monitored quarterly for GWD over one year to determine whether *N. psidii* had spread through roots to the adjacent trees either side of the inoculated tree in the three tree

replicates. Trees showing early GWD symptomatology (Stage 1 of the GWD rating scale) were cut down at the soil line (before bark sporulation occurred), removed and tested for the presence of *N. psidii* by tissue isolations from the main trunk, as described earlier, onto PDA as also described earlier.

The remaining stump was painted with a mixture of copper oxychloride (500g), latex paint (1 liter) and 100ml Bayfidan® made up to 3 liters with water, to inhibit sporulation and finally covered with a black plastic bag, which was sealed at the soil line.

8.3   DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, means were separated using the Waller-Duncan *k*-ratio *t* test (Little and Hills 1990).

8.4   RESULTS

8.4.1 Soil inoculations

Greenhouse soil inoculation with *N. psidii* resulted in significant development of GWD over the six month test period as compared to the Control plants (Table 8.1). These experiments indicated a high potential for rhizosphere infection when root wounding is evident, as usually found in commercial orchards.

Table 8.1. Rhizosphere inoculation of greenhouse clonal guava plants with *N. psidii*

(g)[x]	Elapsed time period between treatment and the percentage of GWD symptomatic plants. Days						Shoot dry mass
	30	60	90	120	150	180	
Experiment 1.							
N. psidii	0 a	30b	30b	30b	80b	80b	
Control	0 a	0 a	0 a	0 a	0 a	0 a	
Experiment 2.							
N. vermoesenii + *N. psidii*[y]	0 a	10b	30b	40b	70b	100b	-
N. vermoesenii	0 a	0 a	0 a	0 a 0 a	0 a	0 a	13.4 b
Control	0 a	0 a	0 a	0 a	0 a	0 a	8.5 a

[x] Mean dry shoot mass assessed at 90 days. [y] *N. vermoesenii* was inoculated into the rhizosphere followed by *N. psidii* 48 hours later.
Figures in each experiment followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.01).
Ten single tree replicates per treatment. Each experiment was repeated twice and the figures above are the means of the two data sets.

Inoculation of the rhizosphere of greenhouse guava plants with *N. psidii* resulted in the development of GWD in 80% of the test plants (Table 8.1) as compared to the Controls where GWD did not develop. *N. vermoesenii* inoculated onto the rhizosphere prior to inoculation with *N. psidii* did not induce any adverse effects on the guava plants. The treatment failed to protect the plants against infection with *N. psidii*. However, *N. vermoesenii* alone did induce a statistically significant growth response in terms of new shoot dry mass three months after inoculation when applied to the roots (Table 8.1 and Fig. 8.1).
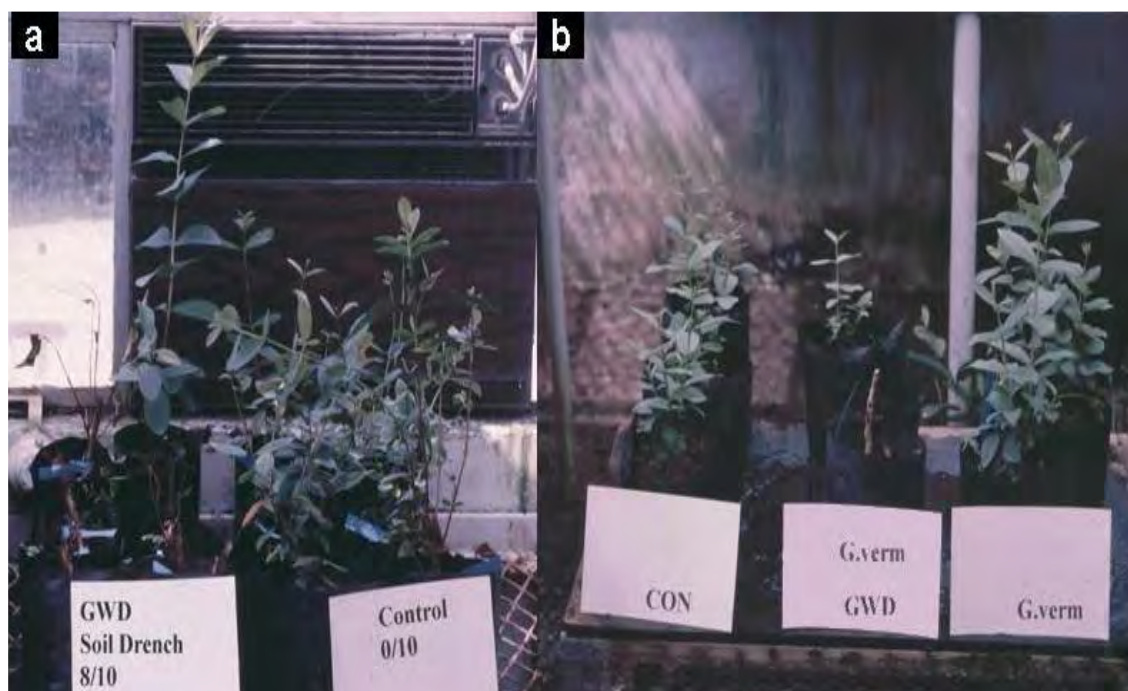
Fig. 8.1. The effect of soil drenches of *N. psidii* and *N vermoesenii* on the growth of clonal (cv. Fan Retief) guava cuttings:

a, dead and diseased guavas plants previously inoculated with *N. psidii* (left); uninoculated plants (right);

b, uninoculated (left), inoculated with *N. vermoesenii* followed by *N. psidii* (center);
N. vermoesenii alone (right).

Note the failure of *N. vermoesenii* to protect the plants from infection with *N. psidii*. Also note increased growth of plants inoculated with *N. vermoesenii* compared to the controls.

8.4.2   Field rhizosphere inoculations

Root wounding immediately after the application of the *N. psidii* inocula to the rhizosphere resulted in significantly higher levels of GWD at 180, 270 and 360 days after treatment (Table 8.2). Two of the Control plants became infected with GWD, but at a significantly lower level compared to the other treatments.

Table 8.2. The effect of root wounding on field rhizosphere inoculations of mature guava trees with *N. psidii*

Treatment	Percentage tree mortality				
	0	90	180 (Days)	270	360
Orchard 3					
UW	0a	0 a	0 a	0 a	0 a
W	0a	0 a	0 a	47 b	73 b
C	0a	0 a	0 a	0 a	0 a
Orchard 4					
UW	0a	0 a	0 a	0 a	27 b
W	0a	76b	87b	100c	100c
C	0a	0 a	0 a	13 b	13 a

[x] 15 trees per treatment. Figures followed by the same letter in each column in each experiment do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).
Each experiment was repeated the following season. Those data presented are the mean values of both experiments (conducted in each orchard). All values were rounded up to the nearest whole number.
UW = unwounded; W = wounded; C = control

In Orchards 3 and 4, rhizosphere wounding did result in significantly higher levels of GWD by the 360 day assessment (Table 8.2). However in Orchard 4, some of the replicates in the unwounded treatment did exhibit GWD symptoms by the end of the experiment, as was the case in the Control treatment. The levels, however, were statistically less than in the wounding treatment.

8.4.3 Large containerized plant inoculation studies

N. psidii was isolated from all the removed symptomatic plants. The presence of barriers between plant rows was effective in stopping root anastomosis and reduced the rate of spread and tree mortality rate of GWD (Table 8.3 and Fig. 8.2).

Table 8.3. The effect of root barriers between tree rows on the rate of spread of GWD in containerized guavas

Treatment	0	65	90	185	290
			(Days)		
			Total tree mortality[x]		
Barriers between tree rows.	0	5	11	18	23
No barriers between tree rows (controls).	0	57	85	103	112

[x] Each tray contained 144 plants.
Inoculation with *N. psidii* was to one tree in the extreme south east corner of the tray, by apical trunk inoculation. Treatments in this trial could not be randomized.

Trays with barriers had a tree loss at the end of the experiment of 16% versus 78% for the tray without barriers between the rows. In these large containerized plant inoculation studies, restricting root contact decreased the spread and mortality rate of guava plants compared to the Control (Table 8.3 and Fig. 8.2). In these experiments, where minimal post inoculation pruning occurred (and all wounds were sanitized immediately), disease spread was largely observed to progress by root to root contact (Fig. 8.3a).
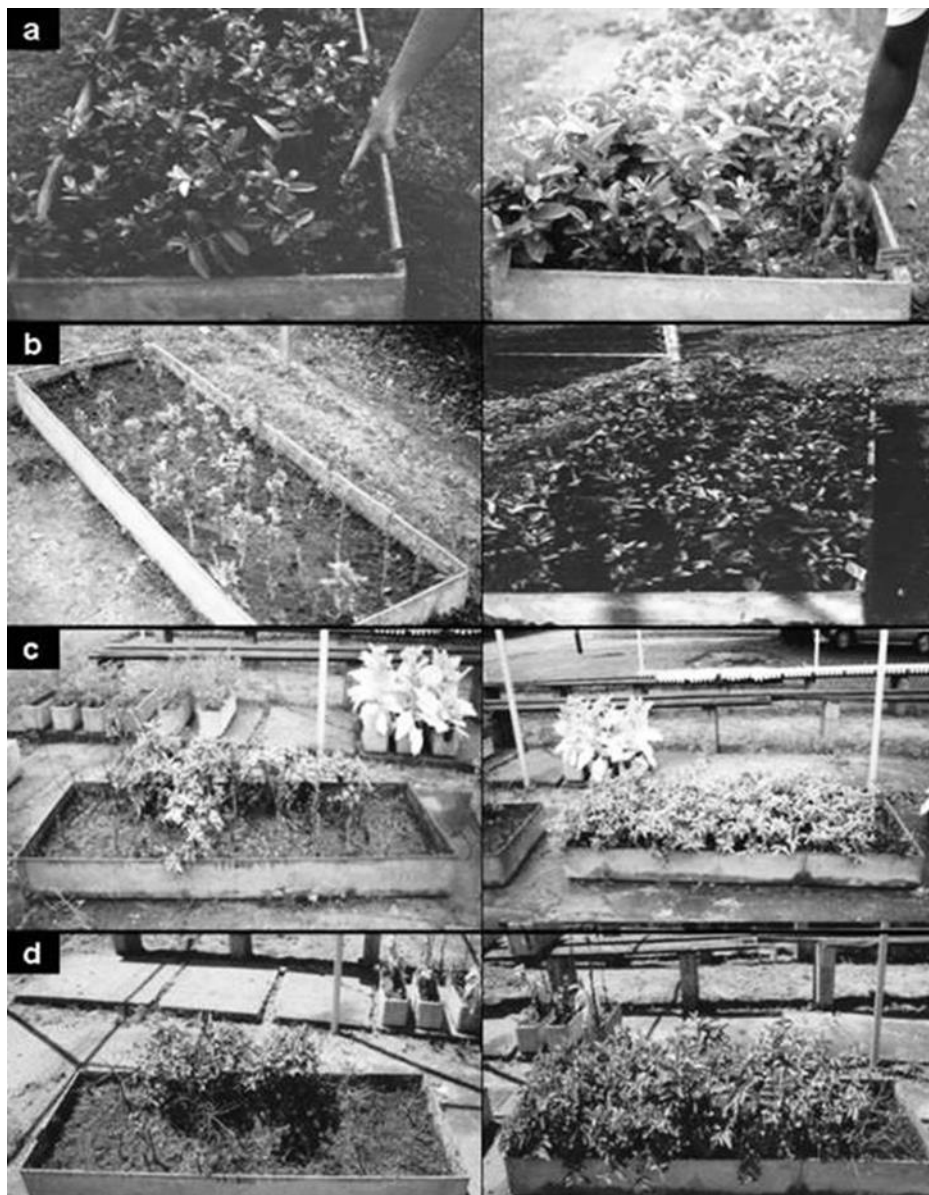
Fig. 8.2. Containerized inoculations of 1 year old clonal (cv. Fan Retief) guava trees:
a, inoculation of one tree in the extreme south east corner of the tray by terminal inoculation of the main stem apex.
a, t = 0; b, t = 65 days; c, t = 185 days; d, t = 290 days.
Right column: plastic barriers between plant rows.
Left column: No plastic barriers between plant rows.

Root contact was observed to occur extensively between trees in the tray without barriers (Fig. 8.3a). Few complete root grafts were detected in these young plants, although root to root contact was widely observed where there was some degree of vascular system contact. Over the course of the experiment, sporulation was not observed on the removed above ground tissues.

Fig. 8.3 a. Root to root contact and anastomoses in containerized clonal guava (cv. Fan Retief) plants as per Fig. 8.2. Healthy tree left in root contact with dead trees right.
b. Field ripping between tree rows and columns to sever root grafting.

8.4.4   Field root contact and anastomosis

Preliminary observations to determine if systemic movement of glyphosate had occurred from a treated tree to an untreated adjacent tree in the row indicated that the affect was not consistent, although symptoms did develop within two weeks of treatment in neighboring trees. Due to the destructive nature of these tests, insufficient replicates could be treated to prove the usefulness of this technique, statistically. However, glyphosate damage was evident on untreated trees next to glyphosate-treated trees and therefore it can be inferred that tree-to-tree contact was successfully shown by this technique. Root grafting was observed to occur at a much higher frequency in older orchards than in younger orchards (Table 8.4). In Orchard 2 (furrow irrigated), widespread evidence of root grafting occurred in the row and, to a lesser degree, across the rows. Root grafting was primarily observed to occur between secondary and tertiary roots.

Table 8.4. The incidence of root anastomosis between trees in guava orchards of different ages afflicted with GWD

	Percentage root graft incidence[x]	
Orchard	In row[y]	Between rows[z]
Orchard 2 (22 years)	70a *	30b **
Orchard 3 (4 years)	0 a	0 a

[x] Roots were observed to determine whether or not they had formed root grafts with adjacent trees.

[Y] Root grafting was assessed between trees in the same row.

[Z] Root grafting was assessed between trees in adjacent rows.

Figures represent the percentage of assessed trees having at least one root graft to its neighboring trees. There were ten trees per treatment.

Means followed by the same letter in each row do not differ from one another according to the Waller-Duncan k-ratio t test (* P=0.01; ** P=0.05).
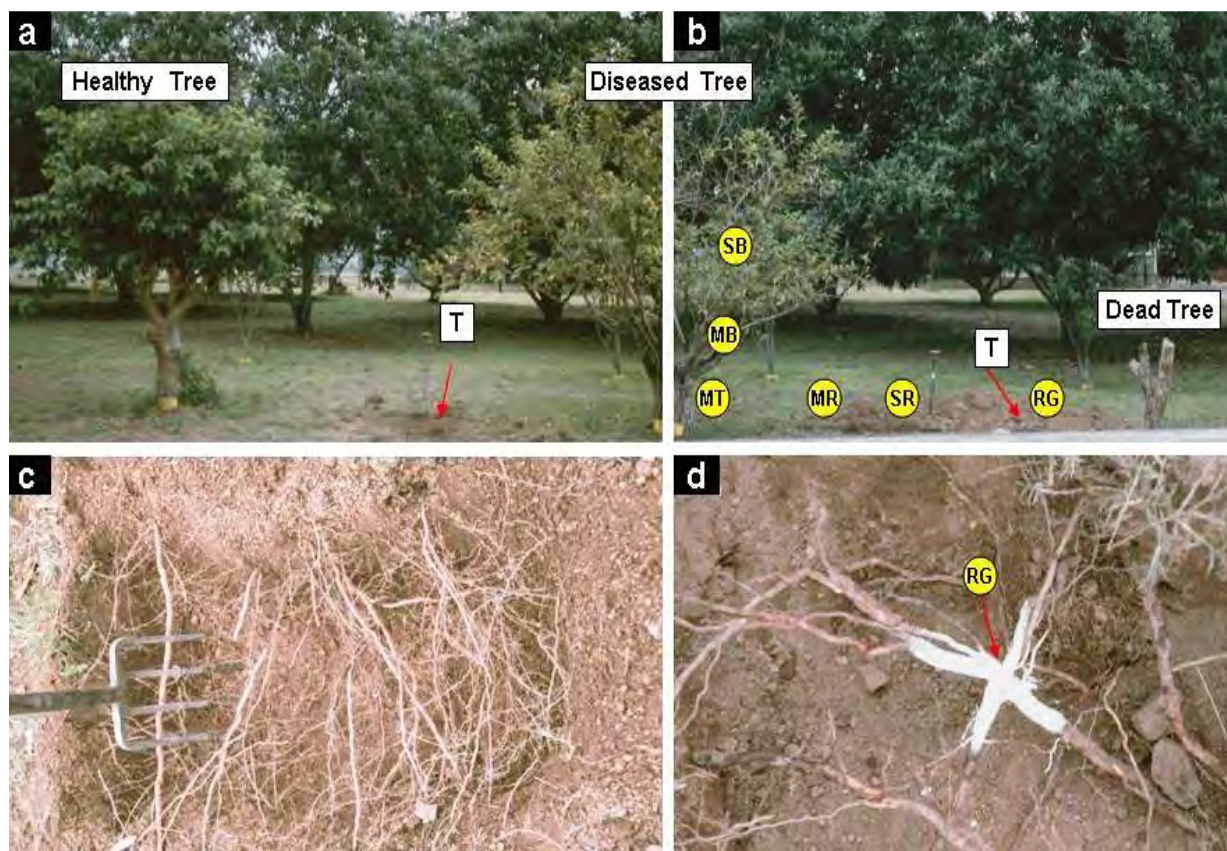
Fig. 8.4. Tree-to-tree spread of *guava wilt* disease by root anastomosis:
a and b, panoramic view of three trees (labelled from left to right; 'Healthy', 'Diseased' and 'Dead') at different disease stages and the incidence of root contact between trees at the same point in time;
c, tertiary root observation in a trench (T), dug in a perpendicular plane to the tree row and equidistant between the healthy tree (1, left) and symptomatic tree (2, center);
d, trench (T) observation of tertiary roots dug in a perpendicular plane to the tree row and equidistant between the symptomatic tree (2, center) and the dead tree (3, right).
Note the presence of root anastomosis (RG).
SR = secondary roots; MR = main roots; MT = main trunk; MB = main branch; SB = secondary branch.

Detailed analysis of a specific example of tree to tree spread in Orchard 2 (Fig. 8.4) confirmed

the presence of root grafting between the dead tree and the symptomatic tree. Tissue sections

of

the symptomatic tree from the scaffold branches to the root graft location confirmed that the

fungus was distributed from the root graft to the main trunk in the symptomatic tree (Fig. 8.5).
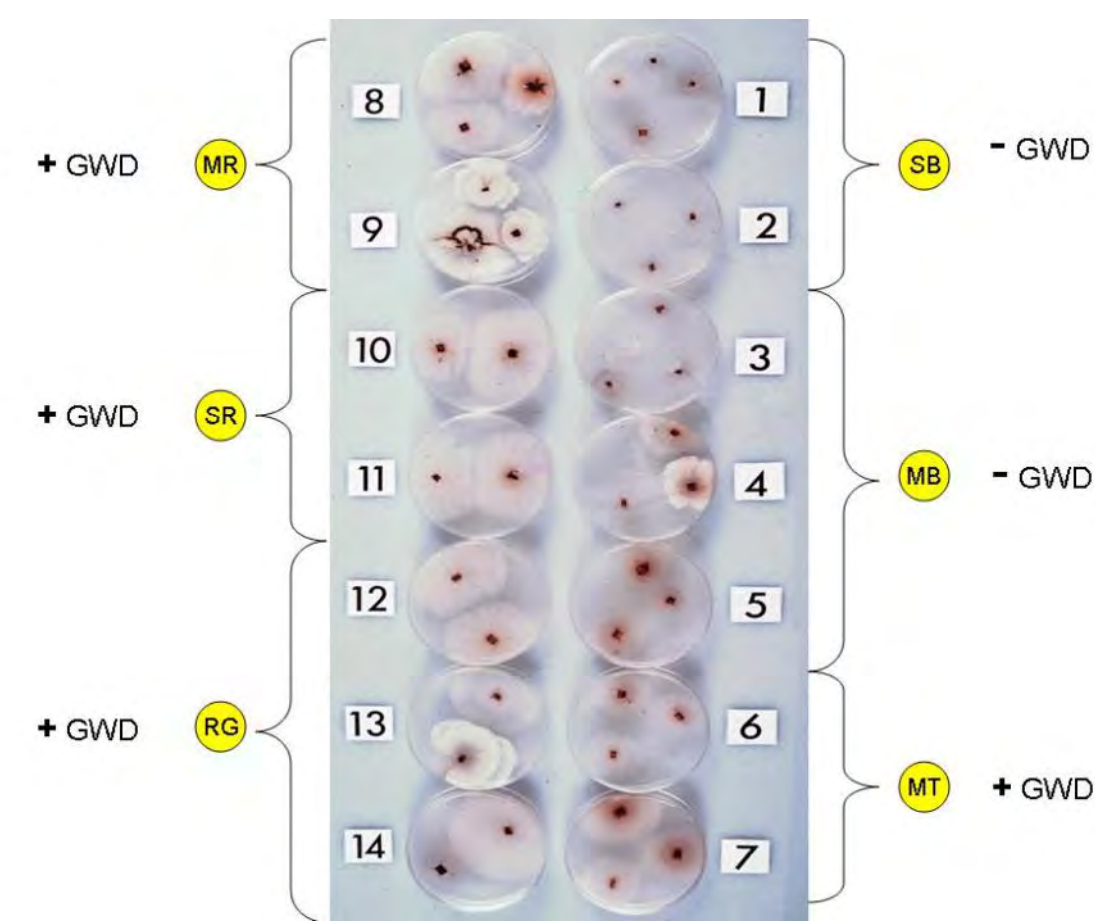
Fig. 8.5. Distribution of *N. psidii* in the vascular tissues of the GWD symptomatic guava tree as shown in Fig. 8.4 (center). Key as in Fig. 8.4.
Tissue sections were extracted from the specimen tree in a numerical sequence commencing with 1 (secondary branches) to 14 (the anastomosed root union between the symptomatic tree and the dead tree). The yellow shaded abbreviations indicate the particular point of tissue extraction for isolation of *N. psidii* as shown in Fig. 8.4. + = tissue is *N. psidii* positive; - = tissue is *N. psidii* negative.

Severing of natural root grafts between trees in the row reduced the tree-to-tree transmission

of *N. psidii* (Table 8.5 and Fig. 8.3b).

Table 8.5. The effect of mechanical severing of natural root grafts between field guava trees

on disease spread to adjacent trees

	Spread of *N. psidii* to the immediately adjacent trees in the same tree column over time[x].				
Tree block	Time (days)				Mean tree no. per replicate with GWD symptoms
	90	180	270	360	
1. Inoculated Ripped					
1	0	0	0	1	1.0b
2	0	0	1	1	
3	0	0	0	0	
4	0	0	1	1	
5	0	1	2	2	
2. Inoculated Unripped					
1	0	1	2	2	1.8c
2	0	1	2	2	
3	0	1	2	2	
4	0	2	2	2	
5	0	0	1	1	
3. Control trees Unripped, uninoculated					
1	0	0	0	0	0.6a
2	0	0	1	1	
3	0	1	1	1	
4	0	0	1	1	
5	0	0	0	0	

[x] Each of the five replicate blocks consisted of three trees in the same tree column, of which the center tree was trunk inoculated with *N. psidii* (isolate CSF221).
The incidence of GWD in the adjacent trees is recorded as:
1= one tree showing GWD symptoms or 2= both trees showing GWD symptoms.
Pruning practices were suspended during this experiment. Trees received monthly full cover sprays of Malathion (62.5g a.i./ 100 liters of water) during the bark borer susceptibility period (April- August). Values in the same column followed by the same letter are not statistically different from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).
In the inoculated ripped treatment, infection rates of adjacent trees were significantly less than

the inoculated unripped treatment in Orchard 2 (Table 8.5).

8.4.5   Rhizosphere fungal population composition

Moribund and recently dead guavas had a diverse population of fungal genera associated with the dead feeder roots (Table 8.6). *N. psidii* was not recovered in these experiments. Feeder roots and secondary roots were rapidly invaded with secondary fungi in the latter stages of GWD.

Table 8.6. Microbial recovery from feeder and tertiary roots of deceased guava trees in the field

_____

Major microbial genera isolated[x]	Months after tree death						
	0	2	4	6	8	10	12
Actinomycetes	+	+	+	+	+	+	+
Pseudomonads	++	++	++	++	+	+	+
Bacilli	+	+	+	+	+	+	+
Fusarium sp.	+	+	+	++	++	++	++
Trichoderma sp.	+	++	++	++	++	+	+
Phytophthora sp.	+	+					
Pythium sp.	+	+					
Penicillium sp.	++	++	+	+	+	+	
Unidentified Fungi	+++	+++	+++	+++	+++	+++	+++
Unidentified bacteria	+++	+++	+++	+++	+++	+++	+++

_____

Major fungal and bacteria genera isolated and identified from feeder root pieces (n = 50 root pieces per tree, 4 trees per time interval).
Key:. >+= = present; >++= = detected in 51% or more of tissue isolations; >+++= present in equal to or greater than 75% of tissue isolations.
Values represent the mean of two experiments.

8.5   DISCUSSION

Soil inoculation of greenhouse plants resulted in the development of GWD within 60 days (Table 8.1) and reinforces earlier studies (Chapter 6 and 7) where rhizosphere infection by *N. psidii* led to rapid tree death.

N. vermoesenii had no impact on suppressing GWD but did have an effect on plant growth (Table 8.1 and Fig. 8.1), as assessed by total shoot mass. This effect may be the result of enhanced nutrient acquisition by the roots or perhaps hormonal stimulation of root tissue. Rhizosphere inhabiting microbes can induce positive plant growth effects, and specifically species within the genus *Gliocladium* have been reported (Harman and Kubicek, 2002) to stimulate plant growth. Specific species of *Gliocladium* [of which several species have been recently reassigned to *Nalanthamala* (Schroers, et al., 2005)], are known to solubilize fixed soil phosphorus (Krzysko *et al.*, 1997). Species within *Gliocladium* such as *G. catenulatum* are known biocontrol agents that have been registered by the US Environmental protection agency as biofungicides (Primastop® biofungicide, Kemira Agro Oy, Helsinki, Finland).

Root barriers reduced plant mortality rates from 0.39 (Control) trees per day to 0.08 trees per day (with barriers), which represents a reduction in the daily tree mortality velocity of approximately over 80 % (Table 8.3). As can be seen in Fig. 8.2(d), in the tray with the barriers installed (right), progression of GWD was largely contained to the tree column containing the tree that was initially inoculated.

This finding concurs with the findings of studies on other fungal vascular wilting diseases (particularly those caused by wound pathogens) such as Dutch elm disease where restriction of root contact/grafting can impair the progression of the disease (Anonymous, 1993a).

N. psidii is a wound pathogen and in these experiments, the importance of root wounding in the etiology of GWD is highlighted by the almost complete absence of the disease in field trees where inoculations were conducted in the absence of wounding (Table 8.2). In Orchard 4, it was observed that some trees that received the rhizosphere inoculation without wounding also succumbed to GWD (Table 8.2). At all the mature tree inoculation field sites, no attempt was made to restrict aerial tissue sporulation on moribund or dead trees. Although pruning was suspended in these orchards during the experiments, cultural practices such as grass mowing did continue and it is postulated that these and natural wounding agents in the soil may have contributed sufficient wounding for penetration by *N. psidii*. Natural wounding agents in the soil have been implicated in many root invading pathogenic fungi (Bruehl, 1987). At both the other rhizosphere inoculation field sites (Orchards 3 and 4) inoculation of the rhizosphere with wounding resulted in the highest levels of GWD across treatments, which was also statistically significant. In Orchard 4, low levels of GWD were recorded in the Control treatments. As this orchard had the highest levels of GWD at the onset of the trials, it is presumed that the Controls became naturally infected with *N. psidii*.

This thesis is further supported by the fact that the Control trees afflicted by GWD were those closest to the natural disease front in the orchard (although still approximately 20-30 m distant at the time of the Control trees' demise).

It is postulated that in the case of the inoculated, non-wounded treatment, natural root wounding factors such as soil cracking, rodent damage or nematode infestation may have opened infection courts for *N. psidii*. All these factors have been previously reported as providing access for tree fungal pathogens (Rozsnyay, 1977; Blanchette and Biggs, 1992).

Initial treatment of parts of the guava canopy with glyphosate, utilized as a non-invasive method to determine tree-to-tree root anastomosis, indicated a high level of root grafting in Orchard 2 (Table 8.4). Glyphosate is considered to be fully systemic, to primarily act on the root system of plants, and to be inactivated when it comes into contact with the soil (Torstensson and Aamisepp, 1977). Therefore, use of this chemical should have been effective in assessing the degree of root grafting in guava orchards. The utilization of this non-invasive technique in determining field root anastomosis may have value as a diagnostic tool when designing field control strategies for GWD where the severance of tree to tree root contact is an important component of GWD management. Further studies on dosage, dilution, seasonal time of application, deployment area on the recipient tree and duration of the response are required to refine this technique and mitigate against excessive phytotoxicity.

Field observations of roots in excavated soil trenches confirmed the preliminary finding that root anastomosis and root to root contact was found to be common in older guava orchards (Table 8.4), particularly between primary and secondary roots (Fig. 8.4). Disease transmission between adjacent mature root grafted trees where the primary and secondary root grafts were carefully exposed by excavation and monitored non-destructively, indicated tree to tree transmission of *N. psidii* primarily through one major root. Feeder roots did not appear to fully

form root anastomoses but did form discreet bundles or ropes (Fig. 8.3a) that were extensively intertwined and easily damaged when lateral shear stresses were applied to them. Detailed investigations in Orchard 2 clearly indicated the presence of numerous root grafts, not only in the row but between the rows (Table 8.4). This observation is consistent with root grafting accounting for the non-random disease spread reported in field epidemiological studies described in Chapter 5.

A specific example of the role of root anastomosis in the spread of GWD is illustrated in Fig. 8.4. Detailed isolations determined that the origin of the *N. psidii* infection in the symptomatic tree, most likely originated from the root graft and progressed to the main trunk only (Fig. 8.5). *N. psidii* could not be recovered from tissues above the main trunk in symptomatic trees, indicating that the pathogen had not moved into the canopy tissues at the time of analysis.

A reduction in *N. psidii* spread was achieved in field experiments where natural root grafting (Figs. 8.3b and 8.4) was severed by sub-soil ripping (Table 8.5). Inoculated plots that where ripped, exhibited statistically reduced levels of infection as compared to the inoculated, unripped treatments. Trees that died as a result of GWD in this experiment were cut back to a stump and painted with a general biocidal mixture to inhibit sporulation and hence it was unlikely that aerial sporulation had any role in subsequent tree to tree spread. However there remained a possibility of root sporulation and spread to adjacent trees. Previous experiments in these studies have clearly shown the possibility of *N. psidii* infection through the soil (Table 8.1-8.3), with and without artificial wounding. However the observation that trees that received ripping treatments (and as such, incurred more root damage than non-ripped trees), exhibited

less *N. psidii* infection would indicate that the severing of root grafts was the primary factor involved in the reduction in the spread of *N. psidii.* Additionally, as all pruning practices were suspended and bark borers were chemically controlled, it is believed that transmission primarily occurred due to root to root transmission, as observed in other diseases (Kentz and Riker, 1950; Yount, 1955; *Appel et al.,* 1989). Although the experimental trees were far removed from the initial disease front at the onset of the experiment, GWD after 270 days was detected within 20 m from experimental trees. Natural spread may have had accounted for some infection in these treatments, but the clear separation between the inoculated treatments that were ripped versus or unripped underscores the important role root anastomosis plays in etiology of this disease.

The general abundance of root grafting in mature commercial guava orchards is an important finding in terms of how *N. psidii* spreads and how the disease could be managed. Strategies to minimize root contact and or root grafting may be useful as part of a strategy to limit the 'in orchard' spread of *N. psidii* and increase orchard longevity. Root barriers are advocated by several authorities as a management component of Dutch elm disease (Anonymous, 1993a). As in guava wilt, persimmon wilt, a disease of similar etiology and caused by *Nalanthamala diospyri*, also aggressively spreads by root grafting (Templeton and Greaves, 1984; Templeton, 1987). As such, the prevention of root grafting in commercial persimmon orchards infected with *N. diospyri* has also been advocated as part of a management strategy for the disease (Crandall, 1950).

Feeder root analyses indicated rapid colonization with a wide array of soil microorganisms (Table 8.6), many known to have strong saprophytic capabilities. It is believed that the presence

of these competing microbial decomposers and the decreasing substrate base rapidly reduces the soil population of *N. psidii.* In addition, lower soil winter temperatures (sub-optimal for *N. psidii*) may also reduce the rhizosphere competency of this fungus, as has been shown for other vascular wilting fungal pathogens (Hessburg and Hansen, 1986).

A critical finding is not only the extent and methods developed in these studies to assess root grafting, but also the importance of restricting root grafting in mature orchards as a method to prevent tree to tree spread. Sequential disease transmission between adjacent mature root grafted trees indicated primary transmission of *N. psidii* via primary and secondary root grafts, and supports previous results from these studies where statistical modelling clearly indicated the non-random nature of *N. psidii* distribution and spread in guava orchards. The distribution of *N. psidii* in a symptomatic GWD tree, infected through root grafting, where the tree xylem tissues were sequentially sectioned, usually indicated a distinct primary root ingress by the pathogen (Fig. 8.6) leading to extensive sapwood colonization of the tree crown and main trunk. Further colonization of the primary root system and egress from the tree crown by *N. psidii* led to further disease spread to adjacent trees by root grafting and on some occasions the source tree was asymptomatic or in the early stages of GWD as assessed by the Guava Wilt Disease Rating Scale (Chapter 6, Table 6.2),

Root anastomosis is well documented as a mode of tree to tree spread in vascular wilting organisms (Stipes and Campana 1981). Clearly reduced root grafting decreases *N. psidii* spread. How economically viable this treatment is remains to be determined. Aerial infection has historically been viewed as the major pathway of infection (Leu and Kao 1979; Hough and

Kellerman, 1986) and as such, significant emphasis has been placed on developing tolerant lines primarily on the basis of aerial infection resiliency (Vos *et al.,* 1998). Earlier studies (Chapter 2 and 3), as in those detailed here, have clearly shown the importance of root infection as a primary mechanism of orchard infection and spread. Recently, orchard infection and spread were reassessed in Taiwan and those findings concurred with those reported here, that root infection is an important and crucial disease pathway in commercial guava orchards (Hong *et al.,* 2013; 2015). The results reported here clearly provide evidence that in older orchards root grafts are common, and that they are a major pathway by which the fungus spreads non-randomly through orchards.
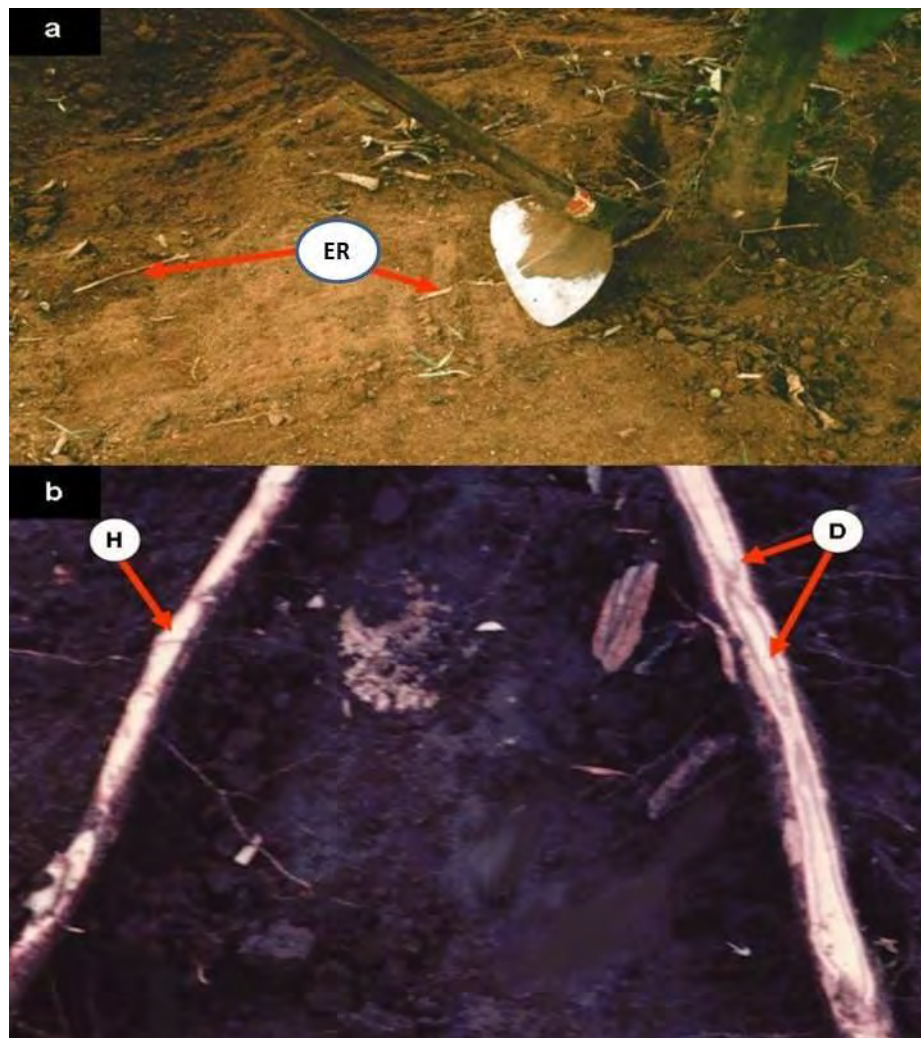
Figure 8.6. *N. psidii* ingress into the guava tree crown via a primary root.

a, Method of excavation and exposure of primary roots between trees utilizing a hand hoe.
b, Healthy primary guava root left, diseased (discolored) primary guava root right. *N. psidii*
was isolated from the root on the right but not from the root on the left. It is interesting to
note that the aerial organs of this tree were symptomless when this photograph was taken.
H = healthy; D = diseased; ER = excavated primary root.

A disease cycle was created to represent the different pathogenic phases of *N. psidii* and the

different plant organs that are infected by different infection pathways, as confirmed in these

studies (Figure 8.7).
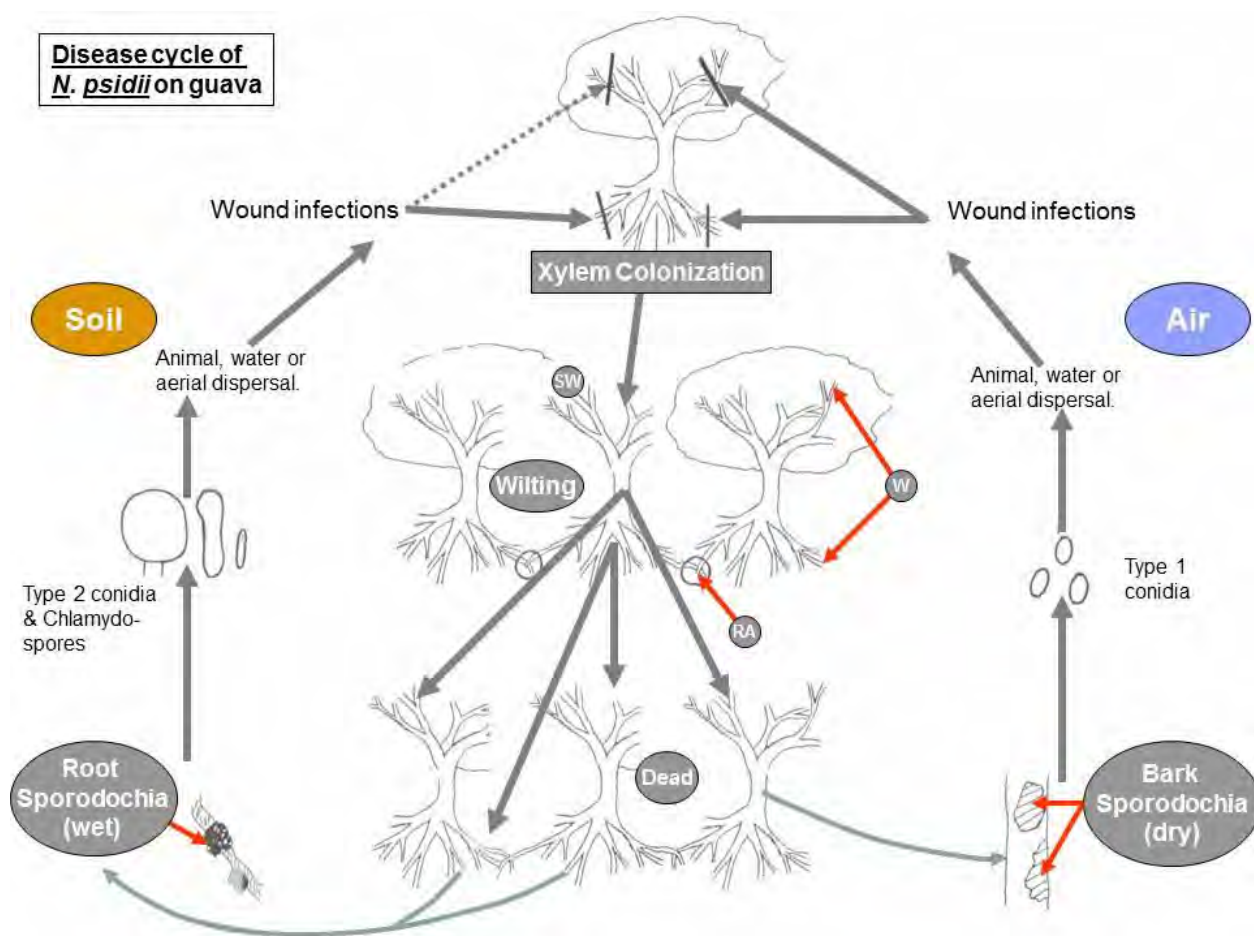
Fig. 8.7. A schematic representation of the pathogenic life cycle of *Nalanthamala psidii* on *Psidium guajava*
W = wound. RA = root grafting/root anastomoses. SW = sectorial wilting.

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CHAPTER 9: HOST RANGE OF *NALANTHAMALA PSIDII* WITHIN THE MYRTACEAE

ABSTRACT

Species from several commercially important representative genera within the Myrtaceae were collected worldwide, imported into South Africa and propagated over several years to generate sufficient germplasm to challenge with *Nalanthamala psidii* (Biourge) Schroers, the causal organism of guava wilt disease (GWD). Plants used in these tests were 7 months to 2 years old. The following plants were challenged with *N. psidii*: *Callistemon citrinus,* bottle brush; *Diospyros virginiana,* American persimmon; *Eugenia pseudopsidium,* wild guava; *Feijoa sellowiana,* pineapple guava; *Leptospernum laevigatum,* tea tree bush; *Myciaria cauliflora,* jaboticaba; *Pimenta doica,* all spice or pimento; *Psidium cattleianum,* strawberry guava; *Psidium friedrichsthalianum,* Costa Rica guava; *Psidium guajava,* common guava (cv. Fan Retief); *Psidium guajava, common guava* (cv. Malherbe); *Psidium montanum,* mountain or spice guava; *Syzygium aromaticum,* clove; *Syzygium jambos*, rose apple*; Eucalyptus* species (*E. nitens, E. cloeziana, E. fastigata, E. deanei, E. grandis* and *E. globulus*). Inoculation of plant material was conducted under controlled greenhouse conditions at 28°C. Most plants challenged with *N. psidii* by main stem inoculation were found to be resistant to the GWD pathogen, although the fungus was frequently re-isolated from the wound site of these resistant plants 90 days after the inoculated pathogen challenge. The following plants succumbed to *N. psidii* inoculation: *M. cauliflora*, *P. guajava*, *E. pseudopsidium,* and *P. montanum*. Two resistant species of the genus *Psidium, P. cattleianum* and *P. friedrichsthalianum,* were graft compatible with Fan Retief. *N. psidii* could be re-isolated from all the *Eucalyptus* inoculation wounds after 90 days. 7 month old plants of *Eucalyptus* species exhibited varying degrees of susceptibility to *N. psidii*, particularly *E. nitens, E. cloeziana and E. deanei*, where all inoculated plants died. All species of *Eucalyptus* were found to be susceptible to leaf inoculation with *N. psidii*, but as with the common guava, this inoculation method never resulted in systemic colonization of the host plant or plant death. 2 year old clones of the *Eucalyptus* species were less susceptible to *N. psidii*, but mortality was still recorded at low levels in both *E. nitens* and

E. deanei, both important commercial species. However, to date there has been no record of mature, field planted *Eucalyptus* being infected or dying as a result of *N. psidii* infection.

9.1   INTRODUCTION

The Myrtaceae contain many economically important genera including the genus *Eucalyptus*. There have been some previous reports of suspected infection of *Nalanthamala psidii* (Biourge) Schroers on species of *Eucalyptus* (Benade *et al.*, 1992) when seedlings were artificially inoculated. These studies were limited in scope, did not indicate if the test material was derived from seed or clonal sources and did not attempt to re isolate the pathogen from potentially diseased tissue. In addition to *Eucalyptus*, the Myrtle family has many important fruit and spice species that have unknown tolerance to guava wilt disease (GWD).

To date, guava cultivars with effective resistance to this disease are limited and most are not commercially satisfactory in terms of yield and quality traits. Some of the few recent cultivars that have exhibited field tolerance to varying degrees, appear to derive their resistance to *N. psidii* through vertical resistance (gene-for-gene resistance) and this has not been stable in the field (Severn-Ellis *et al.,* 2102). Sustainable management of vascular wilting diseases can only be achieved through the development of durable resistance, as curative control of this type of disease is generally not possible (Yadeta and Thomma, 2013). Certain members of closely related genera and certain species may have potential for rootstock purposes and/or inter-genus/inter-species hybrids.

The aims of these studies were to investigate the tolerance of certain type species of important members of the Myrtle family to *N. psidii* under controlled greenhouse conditions. In addition, closely related genera and species to *Psidium guajava* were screened for GWD tolerance, with a view that they may be useful in rootstock development or inclusion in future breeding programs.

9.2   MATERIALS AND METHODS

9.2.1   Alternative host susceptibility of certain species within the Myrtaceae to *N. psidii*.

Clonal propagation material (buds, tissue culture, graftwood, air layers) of several commercially important representative genera within the Myrtaceae were collected from various sources globally and deposited at the Institute for Tropical and Sub-Tropical Crops in Nelspruit, RSA. Plant material was clonally propagated by various methods (rooted cuttings, leaf cuttings, grafting, budding, tissue culture, air layering), predicated by the particular growth requirements of the various species being multiplied. The propagation phase of this particular study was undertaken over the course of 3-4 years to ensure adequate plant material for the establishment of meaningful experiments. The representative species within the Myrtle family were:

a.   *Callistemon citrinus,* bottle brush;

b.   *Diospyros virginiana,* American persimmon;

c.   *Eugenia pseudopsidium,* wild guava;

d.   *Feijoa sellowiana,* pineapple guava;

e.   *Leptospernum laevigatum,* tea tree bush;

f.   *Myciaria cauliflora,* jaboticaba;

g.   *Pimenta doica,* all spice or pimento;

h.   *Psidium cattleianum,* strawberry guava;

i.   *Psidium friedrichsthalianum,* Costa Rica guava;

j.   *Psidium guajava,* common guava (cv. Fan Retief);

k.   *Psidium guajava, common guava* (cv. Malherbe);

l.   *Psidium montanum,* mountain or spice guava;

m.   *Syzygium aromaticum,* clove;

n.   *Syzygium jambos*, rose apple;

Most of the plants listed above were propagated clonally by air-layering or by mist bed propagation of leaf or softwood cuttings, in accordance with Hartmann *et al.* (1983). Plants were planted in 30 cm high plastic bags containing a standard nursery potting mix, as described previously. Plants were grown to a height of approximately 40-50 cm. Four plants of each different species were inoculated with *N. psidii* (Strain CSF221) by the 'bark flap' trunk inoculation method, as described earlier in Chapter 2. A 5mm diameter plug of mycelium from 5 mm behind the advancing front of the expanding colony of *N. psidii* on PDA was removed. These plugs were inserted into a $2cm^2$ bark flap on the trunks, 10 cm above the soil line, of the test plants. The wound was gently wrapped with Parafilm® (Pechiney Packaging Inc., New Jersey, USA, formally American National Can, Chicago, USA), followed by sealing with masking tape (3M, Minnesota, USA), as shown in Fig. 2.1, Chapter 2. At plant death, tissue isolations were attempted to recover *N. psidii*, whereas the inoculation wound in the surviving

plants was assessed at the end of the 90 day challenge, to determine if the fungus could be recovered.

An additional four plants were inoculated by initially conducting a light pruning of the above ground canopy. The main shoots of the test plants were pruned back to within approximately 5cm from the main trunk utilizing sterilized secateurs. Immediately following pruning, a spray of a conidial suspension (approximately 10^6 of a mixture of Type 1 and Type 2 conidia per ml), were applied to the pruning wounds (approximately 1 ml/wound). Each plant had no fewer than 3 pruning wounds. All plants were incubated in the greenhouse at 28°C under natural lighting conditions and monitored weekly for any characteristic symptoms of GWD over the course of 6 months.

In addition to the above species, due to the importance of the genera, six species of *Eucalyptus* (*E. nitens, E. cloeziana, E. fastigata, E. deanei, E. grandis* and *E. globulus*) were screened for susceptibility to *N. psidii* as described above, utilizing 7 month old and 2 year old rooted cuttings. The two year old rooted cuttings were also subjected to leaf inoculations as described in Chapter 7 and comprised of wounding the lower leaf surface by inflicting three to five scalpel cuts, to a depth of 1.0 to 2.0 mm, approximately 5.0 to10.0 mm in length, diagonally across the long leaf axis and as such damaging vascular tissues (Fig. 7.2, Chapter 7). In addition, *Eucalyptus* species were also inoculated in the main stem as described above and assessed over 90 days for the appearance of GWD symptoms and/or plant mortality. Tissue isolations to recover *N. psidii* were attempted from dead plants or from the wound inoculation site at the end of the 90 day challenge period.

Two year old trees of the following species within the Myrtaceae were tested as possible resistant rootstocks for the common guava: *Syzygium aromaticum* (clove), *Leptospernum laevigatum* (tea tree), *Psidium cattleianum* (strawberry guava), *Feijoa sellowiana* (pineapple guava), *Psidium friedrichsthalianum* (Costa Rica guava) and *Diospyros virginiana* (American persimmon). The main stem of these trees were cut off 5cm above the lowest branch. A 10cm piece of *P. guajava (*cv. Fan Retief*)* hardwood with a minimum of three buds was matched to the diameter of the rootstock diameter. The scion budstick was grafted onto the main stem using an apical wedge graft, and the graft union was wrapped with grafting tape. The scion was wrapped with Parafilm®. Plants were placed in a greenhouse at 25°C. After one month the plants were inspected for bud emergence through the Parafilm®. Two buds were selected per scion graft. After six weeks, the Parafilm® and grafting tape were removed and the graft union was inspected for callus formation between the scion and rootstock. Grafted plants were observed for three months to assess new scionic shoot growth and graft compatibility.

9.3   RESULTS

9.3.1   Alternative host susceptibility of certain species within the Myrtaceae to *N. psidii*.

Myrciaria cauliflora (jaboticaba), *Eugenia pseudopsidium* (wild guava)*, Psidium montanum* (mountain or spice guava) and *Psidium guajava (*cvs. Fan Retief and Malherbe) were found to be susceptible to *N. psidii* (Table 9.1 and Fig. 9.1). Within 3 months, plants of these species had succumbed to GWD. *Syzygium aromaticum* (clove) was observed to have some wood discoloration around the inoculation wounds, but remained alive for the duration of the experiment. *N. psidii* appeared to colonize in and around the inoculation point of cloves, but

did not move systemically within the plants' woody tissues.

Table 9.1. Susceptibility of selected species within the Myrtaceae to *N. psidii*

	Wound re-isolation[x]	Plant death (days)[y]	Graft compatible
a, *Myrciaria cauliflora* (jaboticaba)	Y	**50% (90 days)**	N
b, *Psidium guajava* (common guava, cv. Fan Retief)	Y	**100% (40 days)**	**Y**
c, *Syzygium aromaticum* (clove)	Y	N	N
d, *Leptospernum laevigatum* (tea tree)	N	N	N
e, *Eugenia pseudopsidium* (wild guava)	Y	**80% (75 days)**	N
f, *Psidium cattleianum* (strawberry guava)	N	N	**Y**
g, *Callistemon citrinus* (bottle brush)	N	N	N
h, *Feijoa sellowiana* (Pineapple guava)	Y	N	N
i, *Psidium guajava* (common guava, cv. Malherbe)	Y	**100% (60 days)**	**Y**
j, *Diospyros virginiana* (American persimmon)	Y	N	N
k, *Syzygium jambos* (Rose apple)	Y	N	N
***Psidium montanum* (mountain or spice guava)**	Y	**100% (90 days)**	N
l, *Pimenta doica* (all spice or pimento)	N	N	N
m, *Psidium friedrichsthalianum* (Costa Rica guava)	N	N	**Y**

Four plants inoculated per species.

 [x] Isolations were made from the wound site of each inoculated tree to detect the presence or absence of *N. psidii*.

[y] The first figure is the tree mortality expressed as a percentage of the total number of plants. The figures in parentheses represents the average time in days for plant mortality to occur.
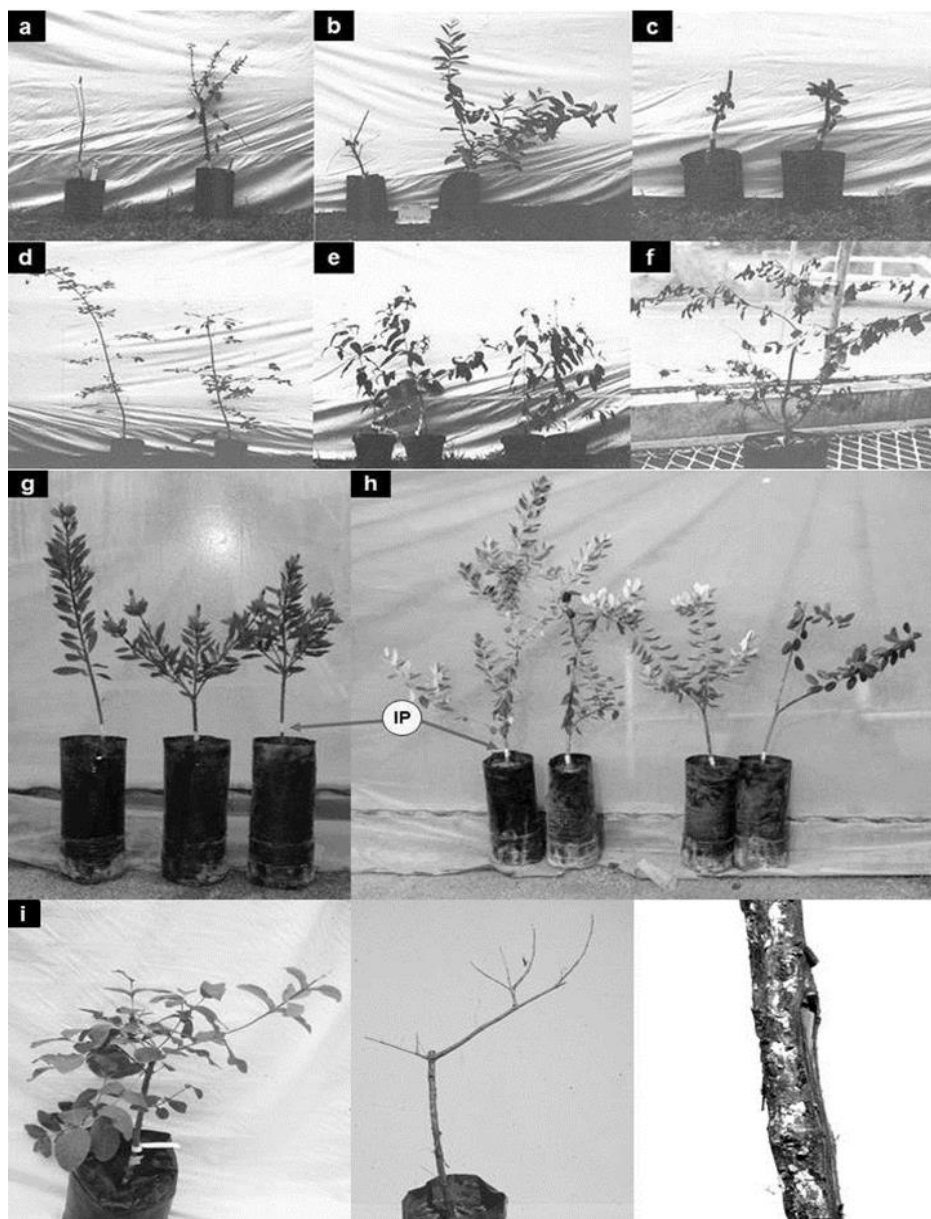
Fig. 9.1. Susceptibility of plants of commercial importance within the Myrtaceae to the guava wilt pathogen: a, *Myrciaria cauliflora* (jaboticaba), susceptible; b, *Psidium guajava* [common guava (cv. Fan Retief)], susceptible; c, *Syzygium aromaticum* (clove), partial susceptibility; d, *Leptospernum laevigatum* (tea tree), resistant; e, *Eucalyptus grandis* (grand gum or rose gum), resistant; f, *Psidium cattleianum* (strawberry guava), resistant; g, *Callistemon citrinus* (bottle brush), resistant; h, *Feijoa sellowiana* (pineapple guava), resistant; i, *Psidium guajava* (cv. Malherbe), susceptible (left = healthy; center= dead; right = stem sporulation). IP = inoculation point.

Leptospernum laevigatum (tea tree), *Pimenta doica* (allspice or pimento), *Diospyri virginiana (American persimmon), Psidium cattleianum* (strawberry guava), *Psidium friedrichsthalianum* (Costa Rica guava), *Callistemon citrinus* (bottle brush) and *Feijoa sellowiana* (Feijoa guava) were all resistant to GWD.

The susceptibility of *Eucalyptus* species to *N. psidii* was found to be higher in younger test plants than older two year old cuttings (Table 9.2). In all cases, *N. psidii* could be isolated from the wound site at the end of the experimental period. Leaf inoculations produced leaf lesions and several days later leaf drop occurred on the seven month old trees, across all species tested. Plant death occurred to varying degrees within the tested seven month old *Eucalyptus* inoculated plants, with *E. nitens, E. cloeziana* and *E. deanei* being the most susceptible (Table 9.2, Figs. 9.1 and 9.2). Two year old trees were far less susceptible, with only *E. nitens* and *E. deanei* exhibiting a low level of tree death (Table 9.2). Leaf lesion size and leaf drop in the inoculated leaves of two year old plants were also much lower than the seven month old trees. *N. psidii* could be re-isolated from all the *Eucalyptus* inoculation wounds after 90 days.

Psidium cattleianum and *P. friedrichsthalianum* were found to be the only species tested that were graft compatible with *P. guajava* (Table 9.1). Grafted greenhouse plants were observed to grow normally over three months, after which they were planted out into the both GWD infected and uninfected fields, for further observation.

Table 9.2. Susceptibility of selected species within the Genus *Eucalyptus* to *N. psidii*

	Wound re-isolation[x]	Plant death[y]	Leaf Inoculation[z] L.L.D.	L.D.
E. grandis				
7 month old cuttings	Y	Y (25)	23	52.5
2 year old cuttings	Y	N (0)	15	27.5
E. nitens				
7 month old cuttings	Y	Y (100)	34	60
2 year old cuttings	Y	Y (25)	28	62.5
E. cloeziana				
7 month old cuttings	Y	Y (100)	38	45
2 year old cuttings	Y	N (0)	11	17.5
E. deanei				
7 month old cuttings	Y	Y (100)	29	70
2 year old cuttings	Y	Y (25)	14	20
E. fastigata				
7 month old cuttings	Y	Y (25)	31	62.5
2 year old cuttings	Y	N (0)	<5	20
E. globulus				
7 month old cuttings	Y	Y (25)	26	40
2 year old cuttings	Y	N (0)	<5	15

Four plants inoculated per species.

[x] Isolations were made from the wound site of each tree to detect the presence or absence of *N. psidii*.

[y] The figures in parentheses are the tree mortalities expressed as a percentage of the total number of plants that were inoculated.

[z] L.L.D. = mean leaf lesion diameter (mm) at 5 days;

L.D. = Leaf drop at 7 days, expressed as a percentage of the total number of inoculated leaves. 10 leaves inoculated per plant.
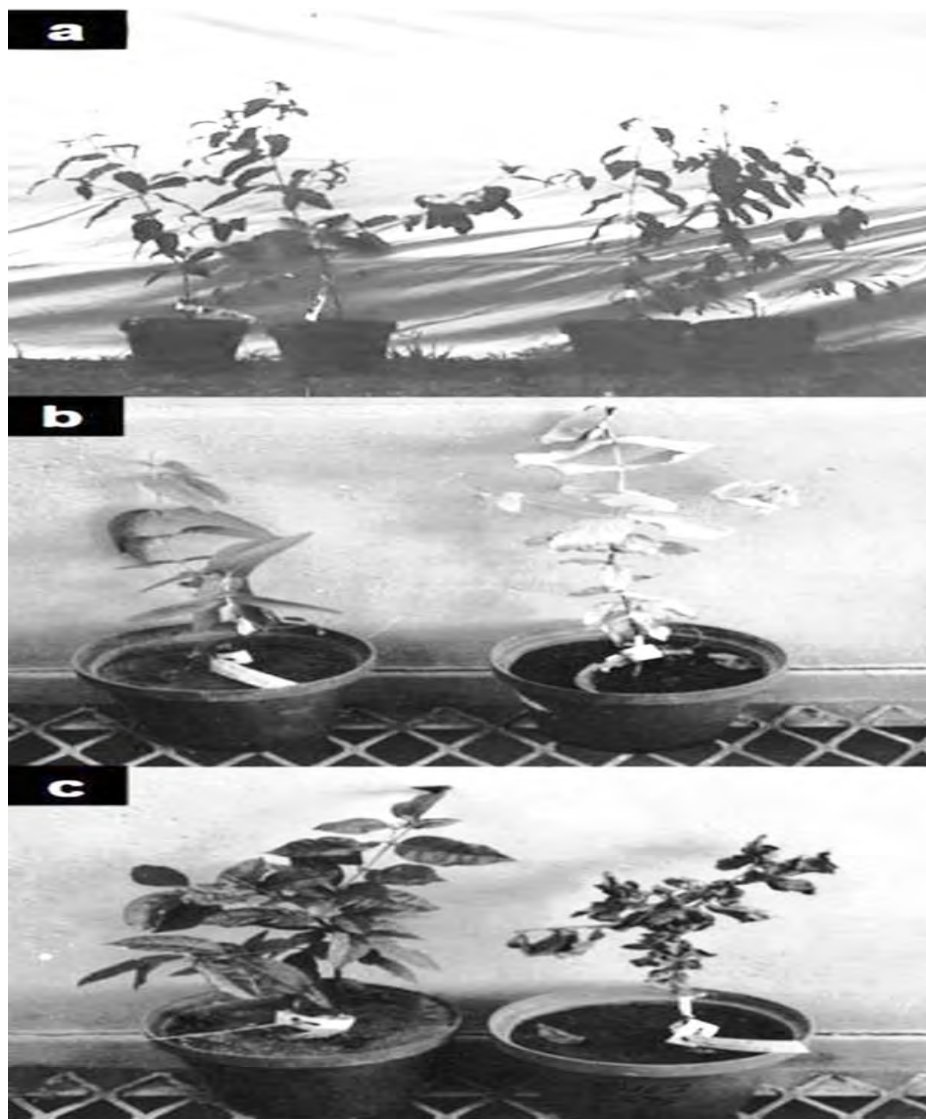
Fig. 9.2. Susceptibility of plants of commercial importance within the Myrtaceae to the guava wilt pathogen: a. *Eucalyptus grandis*, partially susceptible; b. *Eucalyptus nitens*, susceptible; c, *Eugenia pseudopsidium*, susceptible.

Plants were inoculated in the main stem with *N. psidii* (right).

Plants of both *Eucalyptus* species were 7 months old, and the Eugenia species was 2 years old, when these pictures were taken.

9.4 DISCUSSION

Within the genus *Psidium*, *N. psidii* induced mortality in *P. guajava* and *P. montanum* (Table 9.1). Other representative species tested, of commercial importance within the Myrtaceae, with the exception of jaboticaba (*Myrciaria cauliflora*) and the wild guava (*Eugenia pseudopsidium*), were not susceptible (Figs. 9.1 and 9.2). *N. psidii* could be re-isolated from the inoculation sites of the American persimmon (*Diospyros virginiana*), the rose apple (*Syzygium jambos)*, the pineapple guava (*Feijoa sellowiana*) and clove (*Syzygium aromaticum*) but did not induce plant death. *P. cattleianum* and *P. friedrichsthalianum* were not only tolerant to *N. psidii* but were also found to be graft compatible with the common guava over the course of four months from grafting. At the conclusion of these experiments, these grafted plants were planted into both a GWD infected and uninfected orchard and left unpruned. These plants were observed over the course of several years to determine their field tolerance to *N. psidii.* They remained uninfected for a period of 5 years, at which point regular observations of these trees ceased (data unpublished). The use of varieties grafted onto rootstock plants has been successfully used previously to combat soil plant pathogens (Rivard *et al.*, 2012) and in many tree crop industries is a standard practice.

These studies indicated that certain species within the genus *Eucalyptus* were susceptible to the guava wilt pathogen, as was previously suggested by Benade *et al.* (1992). However, in their particular study (where they putatively identified the GWD pathogen as an *Acremonium* species) trees were assessed on the basis of the degree of stem discoloration in response to artificial inoculations without subsequent re-isolation attempts of *N. psidii*. Plant mortality was

not assessed. Our results showed that the susceptibility of *Eucalyptus* species to *N. psidii* was higher in younger test plants as compared to older, two year old cuttings (Table 9.2). In all cases, *N. psidii* could be isolated from the wound site at the end of the experimental period. Leaf inoculations produced leaf lesions and several days later, leaf drop occurred on the seven month old trees, across all species tested. Plant death occurred to varying degrees within the inoculated seven month old *Eucalyptus* plants, but *E. nitens* (Fig. 9.2), *E. cloeziana* and *E. deanei* plants exhibited complete susceptibility (Table 9.2). The finding that species within *Eucalyptus* exhibited greenhouse susceptibility to GWD (Table 9.2) was a most significant finding that could have significant consequences for the South African forestry sector. Fortunately it appeared from these tests that this was largely a phenomenon of very young trees because inoculated two year old trees had much lower levels of plant mortality (Table 9.2). The two most important commercial species (*E. grandis* and *E. globulus*) were largely unaffected (Table 9.2 and Fig. 9.2.). The method utilized in these inoculations (main stem inoculation) is more damaging to younger trees due to the larger proportional impact on their smaller stem diameters. As *N. psidii* was found to persist at the inoculation site of all the screened trees, it is possible that when extensive main stem damage occurs in very young trees, repair and compartmentalization of the infection court is less efficient in juvenile (green) tissues, as suggested by Shigo (1981).

In cooler production regions such as in the Plaston, Brondal, White River and Hazyview regions in the Mpumalanga province, *Eucalyptus* plantations are often found in close proximity to guava orchards. In warmer guava production regions, *Eucalyptus* trees are used as windbreaks and landscape trees. Since the discovery of GWD in South Africa in the early

eighties, no field mortalities of *Eucalyptus* as a result of *N. psidii* infection have been reported. It would therefore seem logical to assume that mature *Eucalyptus sp.* in the field have genetic resistance to *N. psidii* as indicated by the experiments in this study. However, in forests, *Eucalyptus* trees are rarely pruned but are coppiced, and as such, future studies should not only thoroughly assess the impact of *N. psidii* (if any) on mature trees, but also investigate possible infection through this wounding practice.

The reduced basipetal colonization rate compared to the more rapid acropetal xylem colonization rate would suggest why root infections induced plant death more rapidly than aerial infections. This finding together with the determination that *Psidium cattleianum* (strawberry guava) and *Psidium friedrichsthalianum* (Costa Rica guava) are tolerant to *N. psidii* and that they are graft compatible with *Psidium guajava* offers an opportunity to further study the use of these guava types as resistant rootstocks. Both these species have also the benefit of being far more tolerant of the spiral nematode (Grech and Willers, unpublished).

A potentially high impact discovery of this work is the infection potential of *N. psidii* on several *Eucalyptus* species and particularly the two major commercial species; *E. grandis* and *E. globulus*. From these studies it was evident that *N. psidii* can infect young trees (green tissue cuttings) and as such, *Eucalyptus* propagation facilities should take cognizance of this. Infected nursery *Eucalyptus* trees may not be impacted by *N. psidii*, but as shown in these studies, *N. psidii* can persist in *Eucalyptus* tissue and therefore could potentially be disseminated by this route. In many areas of the cooler production regions of the Mpumalanga Province, Eucalypts are often found in close proximity to guavas. The impact of *N. psidii* on older and particularly

forested eucalypts is unclear but warrants further investigation. One aspect of these findings on *Eucalyptus*, is that plant susceptibility to pathogens is not static over the life cycle of the host, nor uniform across all host tissues. As such, durable plant germplasm tolerance screening should acknowledge these variables and incorporate methods of assessment in plant selection programs (Patton 1961; McDonald and Linde, 2002; Bajpai *et al*., 2005; Kamle *et al*., 2012). As seen recently, tolerant guava lines only selected on the basis of aerial *N. psidii* infection of heterozygous populations of open pollinated guava seedlings did not produce durable field tolerance because of root infections (Wan and Leu, 1999, Hong *et al*., 2012, Severn-Ellis *et al.,* 2012).

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CHAPTER 10: EVALUATION OF POTENTIAL BIOTIC SYNERGISTS OF *NALANTHAMALA PSIDII*

ABSTRACT

Nematode surveys of guava orchards in the sub-tropical regions of Southern Africa revealed that the spiral nematode, *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961, was the dominant phytoparasitic nematode. In greenhouse studies the spiral nematode was found to retard guava canopy growth by over 80%. In soils where damaging populations of the spiral nematode were found, soil fumigation not only reduced these populations by 90%, but newly planted guava trees were 250% taller than the controls over a 3 year growth period. Joint inoculations of greenhouse plants (cv. Fan Retief) with *Nalanthamala psidii* (Biourge) Schroers and the spiral nematode, had no effect on guava wilt disease (GWD) development. In the field, soil fumigation and the application of nematicides moderately delayed the onset of GWD at 24 months, but by 36 months there was no difference. The larval stage of two species of the carpenter moth within the genus *Salagena* (*S. obsolescens* Hampson and *S. transversia* Walker), known collectively as the salagena bark borer (SBB), were found abundantly in guava orchards in the sub-tropical regions of Southern Africa during late autumn and winter, inducing sectorial canopy wilting due to bark feeding and wood burrowing damage. The SBB induced canopy symptoms often resemble canopy infection symptoms of GWD. SBB was often found on newly diagnosed GWD afflicted trees. The entomopathogen, *Beauveria bassiana* (Bals.) Vuill. was discovered infecting SBB in tree insect chambers. The fungus was isolated and Koch's postulated fulfilled. Healthy tree inoculations with *N. psidii*, with and without the SBB present, indicated a clear synergy between the organisms in exacerbating GWD. Chemical or biological control (using *B. bassiana*), of the SBB, reduced GWD orchard levels. The association of *N. psidii* and the SBB, shares similarities with the persimmon wilt fungus, *Nalanthamala diospyri* (Crand.) Schroers and its associated bark and wood borers.

10.1   INTRODUCTION

In the absence of obvious wounding agents, guava wilt disease (GWD) caused by *Nalanthamala psidii* (Biourge) Schroers, has been observed to develop rapidly in the field. It is postulated that the many natural wounding processes that the plants are exposed to in nature may account for this rapid disease progress. The development of tree vascular wilts in the field is often exacerbated by biotic and abiotic factors. Abiotic plant stresses in many forms, including water, nutritional, temperature, trauma, and soil and air pollution have been associated with the enhancement of diseases (Bender, 1985; Camps and Bonte, 1991). The severity of the GWD reported from India (induced by *Fusarium oxysporum*), as described by Chattopadhyay and Gupta (1955), has been shown to be substantially influenced by tree age and soil type (Naresh, 1987). Biotic factors associated with fungal wilt diseases are numerous (Bruehl, 1987). Nematodes are frequently associated with increased susceptibility of plants to fungal diseases (Tinsdale, 1931; Neal, 1954; Dropkin 1980; Anonymous, 2008), usually by inciting multiple wound sites (Martin *et al.*, 1956). Nematode feeding causes impaired root membrane function leading to physiological and nutritional stresses (Hamid *et al.*, 1985). These nematode-induced stresses can lead to a modification of plant disease tolerance expression and/or a suppression of systemic acquired resistance (SAR) responses (Powell, 1971). Several nematode genera have been associated with guavas (Anonymous, 1984; Willers and Grech, 1986; Fernandez *et al.*, 1987a; Moura and Moura, 1989; Carillo *et al.,* 1990; Cohn and Duncan, 1990; Khan, 1990).

Bark and wood boring insects have an extensive history of involvement with wilt diseases of trees such as elm (Kendrich and Molnar, 1965), ash (Miller and Hiemstra, 1987), oak (Appel

et al., 1989) and persimmon (Crandall and Baker, 1950; Wilson, 1963).

Guavas are attacked by a variety of wood borers (Holloway, 1986; Sharma and Kumar, 1986; Sandhu *et al*., 1987; Singh., 1988; Singh and Dhamdhere, 1989; Patil *et al*., 1990). Guavas in South Africa are attacked in winter by a metarbelid carpenter moth belonging to the genus *Salagena* (De Villiers and Joubert 2004).Two species are known to occur in Southern Africa; *S obsolescens* (Hanson) *and S. transversia* (Walker). In its larval stage, the moth is a nocturnal bark feeder that bores into the wood where it resides during the daytime hours. Generally these parasites kill tertiary branches by damaging and eventually completely girdling the branch. Due to the severe trunk wounding that these insects induce in guava, and despite the comparative paucity of data, it is clear that wood and bark boring insects could have profound consequences in relation to the spread of *N. psidii*. The aim of this study was to investigate the role parasitic nematodes and the wood boring carpenter moths (both of which are associated with guava), have, in terms of their impact on GWD progress.

10.2   MATERIALS AND METHODS

10.2.1 Nematodes

The spiral nematode, *Helicotylenchus dihystera* (Cobb, 1893) Sher, 1961 was found in preliminary surveys to be the most commonly occurring nematode in guava cultivation in South Africa (Willers and Grech, 1986). Soil samples from guava orchards with a history of nematode infestation were collected to obtain nematodes for plant inoculation studies. Nematodes were collected from infested guava orchards and extracted by a sugar flotation method (Escobar and

Rodriguez-Kabana, 1980; Hooper *et al*., 2005). After extraction, nematodes were surface sterilized with 0.5% hibitane diacetate for 5 minutes, filtered out and re-suspended in SDW. The concentration of nematodes were adjusted to $6000.L^{-1}$ and 0.5 L of this suspension, was applied to two treatment groups. Fifty clonal guava plants (cv. Fan Retief) were grown from cuttings and established in 4 liter plastic plant bags containing methyl bromide ($75g.M^{-3}$) fumigated nursery soil. These plants were allowed to grow for two weeks, after which experimental plants were selected for uniformity. Treatment plants (15) were infested with nematodes by drenching the suspension onto the surface of the soil whilst gently agitating the soil surface with a hand garden fork. In addition four, 10cm deep, 2cm diameter cores equidistant from one another and halfway between the perimeter of the pot and the stem, were made into the rhizosphere with a cork borer. After drenching with the nematode inocula, these cores were filled with sand. After one week, one replicate set of the nematode inoculated plants (and one replicate set of 15 nematode-free plants) were inoculated with a slurry preparation of *N. psidii* containing approximately 7.2×10^6 conidia ml^{-1}, produced and administered in an identical manner to that used previously to inoculate soil with *N. psidii* (Chapter 8). Control trees received 500ml of SDW. Prior to being inoculated, plants were pruned to the same height and the wounds painted with fungicidal suspension comprising of copper oxychloride (250g. L^{-1}) plus latex paint (250g. L^{-1}) in water. Plants were observed over several months for the development of GWD. Trees surviving at the end of the experimental period had all their new growth removed, dried at 70°C to a constant mass and weighed.

10.2.2 Nematode distribution in guava orchards and their effect on GWD development

Further surveys were undertaken to establish the presence, distribution and populations of various nematodes found in guava cultivation (in GWD infected and GWD free orchards) in production regions of South Africa (Mpumalanga and Limpopo Provinces), Swaziland and Mozambique. Composite samples comprising of a 250g mixture of soil and roots, were taken from the rhizosphere of ten trees in an orchard during spring and the end of summer. Plant parasitic nematodes were extracted by standard methods (Escobar and Rodriquez Kabana, 1980; Southey, 1986; Hooper *et al*., 2005). Nematode extraction was accomplished using a combination of the wet sieving technique and sugar flotation (for soil) and the Baermann funnel technique (for roots). Nematodes were counted under the dissection microscope using a haemocytometer. Nematode infestation was also compared on trees at different canopy GWD rating stages (Chapter 6, Table 6.2) where the disease was present.

Orchard 4 (Chapter 5, Table 5.1) was selected due to the high soil spiral nematode counts (>8000 nematodes per liter of orchard soil). Ten trees free from GWD and at least 150 m from the closest infected tree, were placed under a nematicide program consisting of applications every 60 days of a mixture 40 g.m^{-2} Temik$^{®}$ 15G [(aldicarb) Bayer Agrochemicals Ltd, „Johannesburg)] and 40g.m^{-2} Nemacur$^{®}$ 10G [(fenamifos) Bayer Agrochemicals, Johannesburg], applied to the soil under the canopy. Nematicide treatments were applied monthly by hand and spread under the entire tree canopy. During mid-winter (July) to mid-Summer (December), which encompassed the main guava root flushing periods (Grech, unpublished data). Fruit from these treated trees were not harvested and the treated area

signposted with warnings that the fruit must not be eaten. Controls were untreated. Trees were unpruned for the duration of the trial.

In addition, a portion of Orchard 5 (Chapter 5, Table 5.1), where most of the trees had recently died from GWD, was selected, annexed and designated 5(a). The orchard previously had high disease pressure and at the time the trial plot was being prepared, the closest GWD disease front was approximately 200 m distant. A portion of this orchard (approximately 2 ha) was selected and the boundaries were marked by wooden stakes.

Spiral nematode counts at this site were in the range of 4000-5000 nematodes L^{-1} of soil, which was considered moderately damaging (Willers[1], personal communication). Trees were removed (including the stumps and major roots systems), burnt on site and the ashes spread as evenly as possible over the entire test site. The soil was ripped to a depth of 1 meter along the proposed tree planting line as well as perpendicular to the tree lines and intersecting the proposed tree planting stations. Soil analysis was conducted as described by Du Plessis and Koen (1984), and the soil adjusted with amendments and fertilizers, as indicated by the soil analysis. The soil was then ploughed, harrowed and cultivated to a fine tilth. Plant stations were marked and drip irrigation installed with two drippers (each delivering $4L.hour^{-1}$ of water), placed at each plant position. Small plot (20 m x 5 m) replicates were demarcated and labeled. In the fumigated treatments, plots were treated in summer with $70g\ M^{-2}$ methyl bromide using the hot gas method (Anonymous, 1982; Willers and Grech, 1986) and tarped with 50 *u*M thick low permeability plastic. All treatment plots were located at approximately the same latitude

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[1] Willers, P. Senior researcher, ARC-ITSC, Nelspruit, retired.

and spread across the middle of the orchard site in an east-west orientation. The plastic tarp was removed from the fumigation treatments one week post fumigation and the soil allowed to vent for a further two weeks. The demarcated trial area was isolated from the surround by the installation of a 2 meter high chain link fence. Gates were installed at the north and south of the orchard, and kept locked. The aim was to prevent unauthorized access and possible anthropogenic disease spread.

One year old guava plants (cv. Fan Retief) were planted in each of these plots at 2 meter spacings, resulting in 10 trees per plot. Trees were managed culturally under the standard guava farming practices adhered to on the farm which were in accordance with industry guidelines (De Villiers and Joubert, 2004). The only deviation from this was that all the experimental trees were not pruned for the entire experimental period, and weed control was entirely chemically based. Each plot represented one replicate and each treatment was replicated 5 times. In Treatments 1 and 2, trees were biannually (June and October) treated for 3 seasons from planting with $40g.m^{-2}$ Temik® 15G [(aldicarb) Bayer Agrochemicals Ltd, ,Johannesburg)] and $40g.m^{-2}$ Nemacur® 10G [(fenamifos) Bayer Agrochemicals, Johannesburg]. The nematicides were applied uniformly onto the soil under the canopy. When the trees reached approximately two years of age, GWD had reached the northern boundary of the neighboring orchard and at that time the prevailing disease front was still approximately 150M from the closest replicate. Treatment 2 (fumigation, chemical nematicides and *N. psidii*) comprised of applying inoculum of *N. psidii* to the soil surface (as described previously in this Chapter) of plots treated identically to Treatment 1. Treatment 3 was inoculum of *N. psidii* only, applied to untreated soil without wounding. Treatment 4 was soil fumigation alone and in the Control, the soil was untreated. Trees in each plot were assessed every 6 months for the presence of GWD

symptoms. In both field experiments, rhizosphere samples were collected every six months as described earlier in this study, from each tree of each replicate and homogenized into one composite sample per replicate. These samples were analyzed to determine the level of nematodes in the soil. Plant height (soil to the tallest point on the tree) was assessed annually. All fruit from trees receiving nematicides were destroyed. The orchard was monitored for three years.

10.2.3 The influence of the Salagena bark borer (SBB) on GWD

A 0.5 ha GWD free portion of Orchard 3 (Chapter 5, Table 5.1) was sprayed with a motorized agricultural sprayer every 10-14 days commencing in mid-March with a mixture of 1.5 liters citrus spray oil (Dow Agrosciences, Pretoria) and cypermethrin EC 200 (Syngenta, Midrand, South Africa) at a dosage of 50 ml / 100 liters of water. Sprays were directed at the inner canopy and applied to runoff (approximately 4L / tree). These sprays were continued until early May.

Guava trees in GWD free orchards were inspected during May and June for the presence of the Salagena bark borer (*Salagena obsolescens* Hampson and *Salagena transversia* Walker). Trees were marked during the day, and larvae were collected at night, when they emerge from their burrows to feed. These collected larvae were first refrigerated at 8°C for 1 hour. The larva were then surface sterilized by immersion in a chilled (8°C) 0.25% solution of hibitane diacetate (Protea Ltd, Johannesburg) for 20 seconds under gentle agitation, rinsed in SDW and gently dried on sterilized tissue paper. The larva were placed in sterilized sawdust in a vented

container until examination (approximately within 24 hours) and stored at 8°C until required.

The influence of the SBB on the development of GWD was investigated by applying SBB to uninfested guava trees with and without *N. psidii* utilizing a treatment schedule, as outlined in Table 10.5. Seven days prior to placing the SBB larva onto guava secondary branches, the inner branches of the trees were treated with a high pressure spray of 125mM sodium hydroxide and 0.03% Tween 20® in 100 liters of water to deactivated and wash off recalcitrant resident insecticide residues. Three hours later the treated branches were pressure washed with water. The moth was applied before or after application of *N. psidii*. The insects were applied by introducing them onto uninfested, GWD-free mature trees (all treatment trees were at least 100 m from the nearest GWD infected tree). The insects were deployed in the late afternoon by placing one larva on a secondary branch. These treatment trees were within the 0.5 ha block that had been treated with insecticides for several months prior to the onset of this experiment. Two treated larvae were placed on each tree, each on a separate secondary branch. Additionally, the secondary branch area that the treated larvae were placed on, was treated with a conidial spray of *N. psidii* (5.8×10^{-5} cells ml^{-1}) plus 0.1 % Tween [20], in a band extending 20 cm above and below the larva placement points either prior to or post insect deployment as per Table 10.5. Viability of the conidia in the final dilution were assessed by spraying 5 Petri plates containing acidified water agar. Treated Petri plates were incubated at 28°C in darkness for three days and microscopically assessed for conidial germination. The surface sterilized Control treatment larva were immersed in SDW plus 0.1% Tween[20] momentarily, similarly placed on an uninfested tree and the secondary tree branch further treated with a topical spray of SDW in a 20 cm band above and below the placement point before or after placement. Eight

291

trees were used for each treatment. All the larvae were covered with a wire mesh dome following placement to avoid bird or reptile predation during the first few hours of placement. Activity levels at the infestation sites were checked 48 hours after placement (when the mesh protective domes were removed) and if there was no evidence of frass accumulation around the emergence hole (or the lack of an emergence hole), larvae were presumed dead or absent. Where no activity was observed after 48 hours, new larvae were applied to exactly the same areas and the treatment repeated. Additionally, all trees in these experiments had polybutene ant barriers attached to their main trunks. Trees were monitored over several months for the development of GWD.

10.2.4 Bark borer control and GWD development

Fifteen trees visually free of GWD and at least 50 m away from the nearest GWD infected trees in Orchard 2 were selected during early to mid-Autumn (March-April), and sprayed alternately with 100ml Cymbush® 5EC [cypermethrin (Syngenta Pty, Ltd., Midrand)] and 250g Mercaptothion® 250WP [malathion (Dow Agrochemicals, Pretoria)] on a monthly basis for four months. Both products were diluted in 100 liters of water and the pH adjusted to 6.5, prior to applying a full cover spray (equivalent of approximately 2000 L ha^{-1} spray volume) to experimental trees by a motorized vehicle mounted hand sprayer operating at 150 psi. All sprays contained 0.05 % (v/v) of the spreader/sticker, Latron B® (Dow Agrosciences, Indianapolis, USA). Care was taken to ensure that the whole tree and inner branch canopy was thoroughly covered.

A biological control treatment was applied that comprised of an entomopathogenic fungus that had been previously isolated from SBB larva cadavers from pecan, *Carya illinoinensis* (Wangenh.) K. Koch trees and shown to be pathogenic to these insects (Grech, unpublished, Fig. 10.5). The fungus was cultured and identified by the author as *Beauveria bassiana* (Bals.) Vuill. The fungus was bulk cultured on an autoclaved mixture of ground shrimp shells (25%w/w) and rice (40% w/w), infused with 35% (w/w), half strength, Sabouraud's dextrose broth. The media was placed in autoclavable plastic bags, autoclaved, then inoculated with the entomopathogen before being incubated at 25°C under incandescent illumination (12 hours of light per day) and agitated every two days for two weeks. After incubation, the fungus was removed from the bags and placed in a food blender (Kenwood, USA) with an equal quantity of SDW, and blended for 2 minutes using the 'puree' setting. During the last 10 seconds of blending, the blender was adjusted to the 'stir' setting and 0.1% Tween-20 was added to the fungal suspension. The suspension was filtered initially through cheesecloth and then through a 20 *u*m Millipore filter. The final spore count was adjusted to approximately 5×10^5 conidia ml^{-1}, and 1 L of this suspension was applied in 100 L of water, four times at monthly intervals commencing in April, just prior to dusk. The spray was applied to 15 uninfested trees, plus five additional infested trees (which just prior to spraying had all the frass coverings removed from the SBB burrows). The spray was applied by a hand held spray lance from a motorized, vehicle mounted agricultural sprayer operating at 150 psi. The spray was directed to inside the tree canopy (particularly on the scaffold branches and main trunk). Viability of the conidia in the final dilution was assessed by spraying 5 Petri plates containing half strength Sabouraud's dextrose broth as well as 5 Petri plates containing acidified water agar. Both types of media were modified with 250mg.L^{-1} ampicillin. Treated Petri plates were incubated at 28°C in

darkness for three days to assess conidial germination. Control trees did not receive any treatment. All the trees in this experiment were initially free of GWD and at least 50 M from the closest tree with GWD.

Trees were monitored over one year for the development GWD. If trees died, then standard procedures to detect *N. psidii* were implemented, as described previously (Chapter 2). Normal cultural practices were maintained with the exception of pruning, manual weed control and fungicidal sprays, which were not conducted during the course of this experiment. Bark borer activity was also monitored in the five infested trees by observing the buildup of frass at weekly intervals during the night for a period of one month. Where possible, moribund insects or cadavers were collected from these trees and assessed for infection with *Beauveria bassiana*. Tissue isolations were made onto half strength acidified Sabouraud's dextrose broth, amended with 250 mg.L^{-1} ampicillin.

10.3   DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, means were separated using the Waller-Duncan *k*-ratio *t* test (Little and Hills 1990).

10.4   RESULTS

10.4.1 Nematodes

The spiral nematode (*H. dihystera*) was found to be the most common plant pathogenic nematode associated with guava cultivation in the summer rainfall region of sub-tropical regions of Southern Africa (Fig. 10.1). The effects of this nematode on guava dry shoot mass

was clearly demonstrated in the greenhouse trials (Table 10.1 and Fig. 10.2). At some sites, particularly in the warmer sub-tropical lowland regions of the survey, the spiral nematode was usually found together with the root knot nematode (*Meloidogyne* sp.). These mixed root infestations were observed to induce substantial damage (galling and feeder root death) to the guava root system (Fig. 10.3).
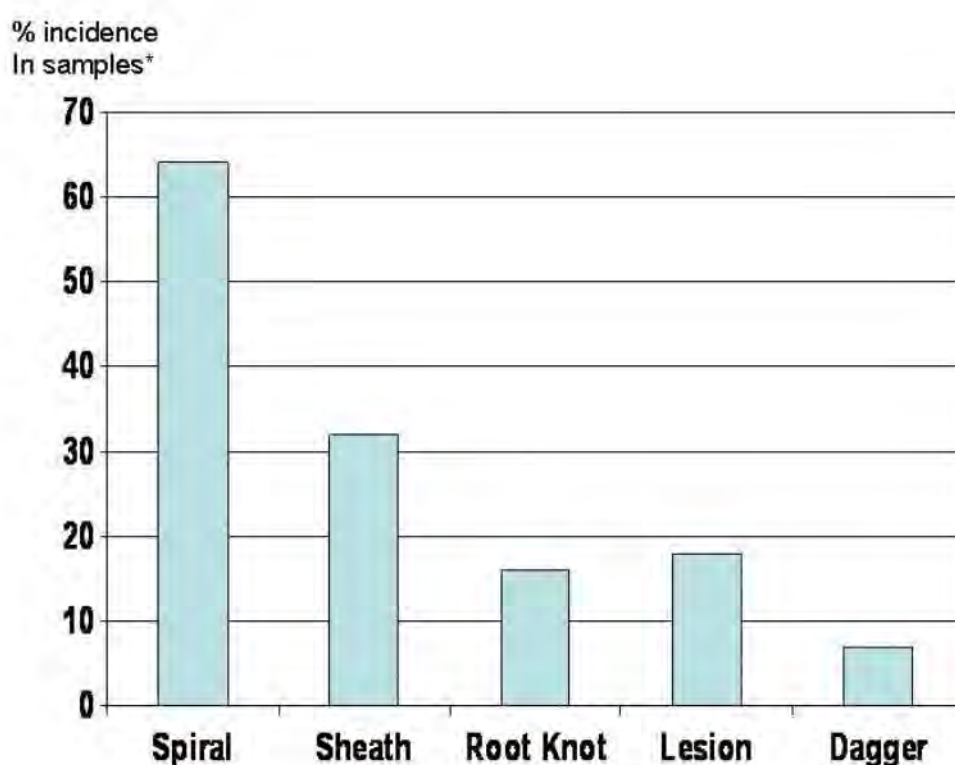
Fig. 10.1. The incidence of plant pathogenic nematodes in soil samples collected in guava orchards in South Africa (Mpumalanga and Limpopo provinces), Mozambique (Maputo province) and Swaziland (Malkerns and Manzini regions). N = 87.
Samples were assessed for the incidence of the specific nematode in each sample.
Values represent the presence of the specific nematode (expressed as a percentage), detected across all the samples analyzed.

However, the absolute level of root damage was not quantified. Other plant pathogenic

nematode genera that were found on guava included: *Pratylenchus* (lesion nematode), *Xiphinema* (dagger nematode) and *Hemicycliophora* (sheath nematode), as illustrated in Fig. 10.1.

Table 10.1. The influence of the spiral nematode, *Helicotylenchus dihystera* on shoot growth of greenhouse guavas and the effect on the development of GWD

Treatments	Elapsed time period between treatments and the percentage of GWD symptomatic plants. Days					Dry shoot mass[x]
	30	60	90	120	150	
Nematodes[y]	0a	0 a	0 a	0 a	0 a	4.6g a*
Nematodes + *N. psidii* soil inoculation[z]	0c	26b	33 b	100b	100b	Dead
N. psidii soil inoculation	7b	73c	100c	100b	100b	Dead
Control	0a	0 a	0 a	0 a	0 a	27.4g b*

[x] Mean dry mass of the shoot regrowth over the experimental period (150 days).
[y] 3000 nematode J2 larva inoculated per plant.
[z] *N. psidii* inoculated one week after nematode applications.
Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05; *P=0.01).
Fifteen single tree replicates per treatment.

Treatment of nematodes by post-plant chemical agents were found to significantly reduce nematode populations by over 80% compared to the Control treatment (Table 10.2). GWD was not present in these experimental plots for the duration of the experiment (Orchard 2).

Fig. 10.2. The effect of the spiral nematode on guava growth and infection with GWD.
a, guava cuttings growing in fumigated soil (background) and guava cuttings growing in fumigated soil artificially infested with the spiral nematode (foreground).
b, guava cuttings growing in fumigated soil infested with the spiral nematode (right) and guava cuttings growing in fumigated soil infested with the spiral nematode together with a *N. psidii* soil drench (left).
Note several plants have died in the nematode + *N. psidii* treatment (t = 41 days).

Table 10.2. The effect of chemical nematicide application on populations of the spiral

nematode, *Helicotylenchus dihystera,* and on the incidence of GWD in the field

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Elapsed time period from the commencement of treatments.

				(Months)				
Treatments	3	6	9	12	15	18	21	24
Temik® and Nemacur®								
Nematode counts[x]		1500a		2200 a		<500a		1300a
GWD incidence[y]	-	-	-	-	-	-	-	-
Control								
Nematode Counts[x]		5500a		11,500b		8500b		9500b
GWD incidence[y]	-	-	-	-	-	-	-	-

_____

Orchard 2 (Chapter 5, Table 5.1).

[x] Nematodes were extracted utilizing the sugar flotation method described earlier in this Chapter.

[y] GWD was assessed across all replicates and recorded as present (+) or absent (-) in the treatment or the control as a whole.

Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Five replicates per treatment.
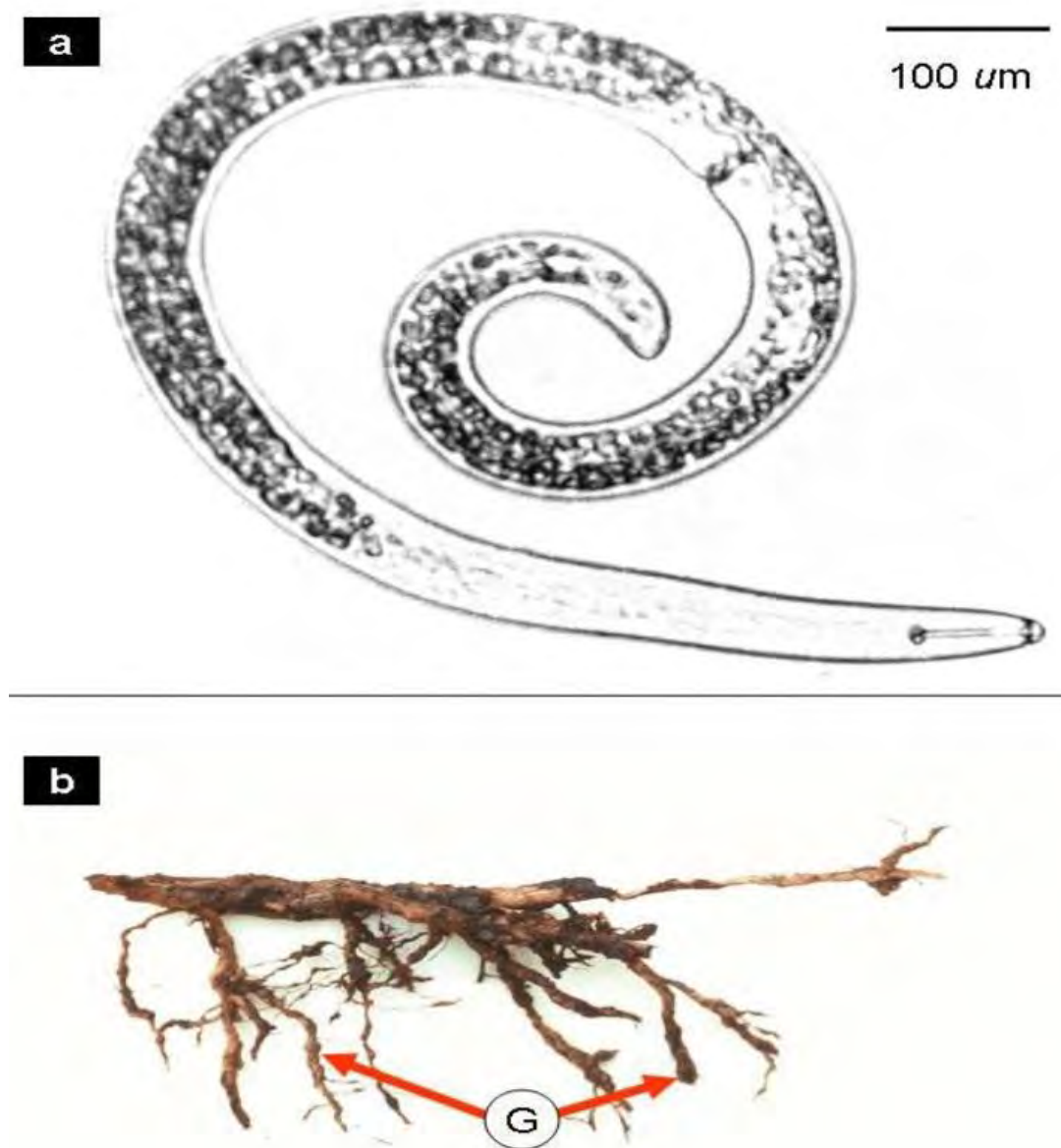
Fig. 10.3. The spiral nematode and nematode induced damage to guava roots.

a, The spiral nematode *Helicotylenchus dihystera* (♀).

b, guava root damage induced by mixed populations of the spiral nematode (*H. dihystera*) and the root knot nematode(*Meloidogyne* sp).

G = root galling.

In Orchard 5(a), pre-plant fumigation, coupled to post-plant treatment with nematicides, resulted in reduced soil nematode levels, and trees that were 250% taller than unfumigated Control trees (Fig. 10.4 and Table 10.3).
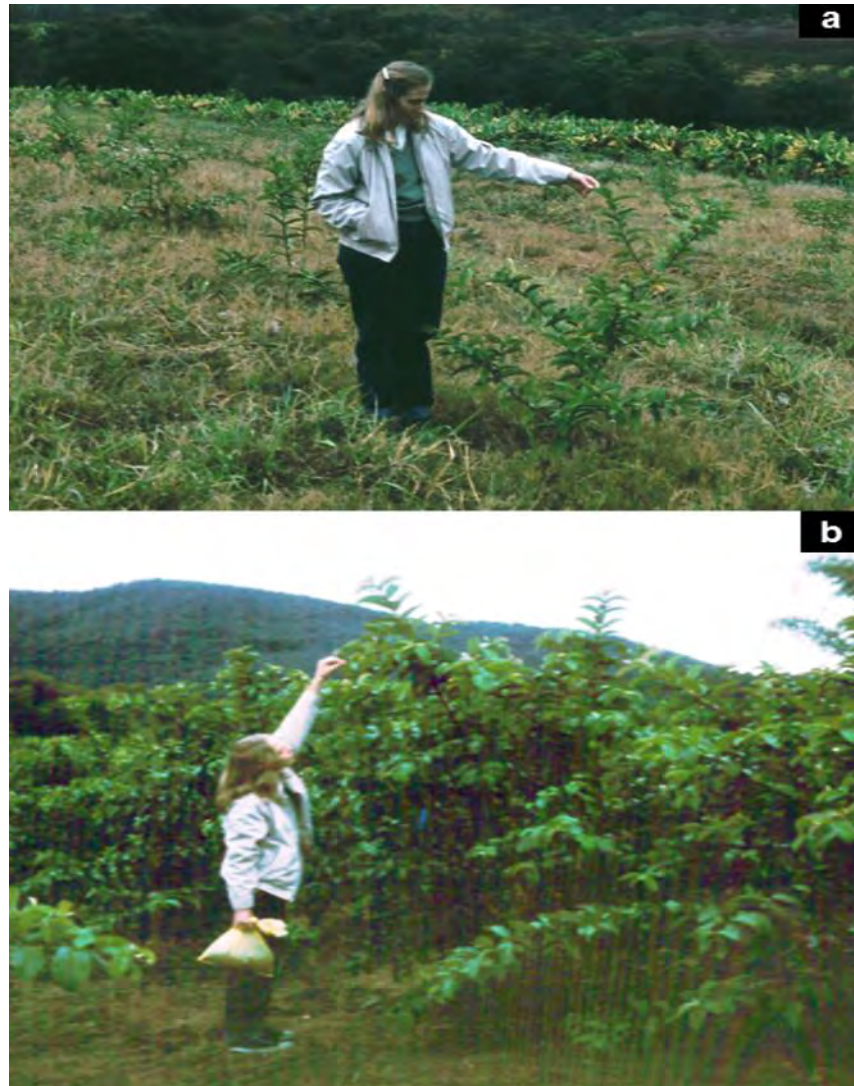
Fig. 10.4. The effect of spiral nematode (*Helicotylenchus dihystera*) infestations on field guava cultivation.
a, 2 year old guava trees that were planted into soil heavily infested with the spiral nematode.
b, 2 year old guava trees planted at the same site as 'a' except that the soil was fumigated with methyl bromide prior to planting.

Table 10.3. The effect of pre plant fumigants, chemical nematicides and *N. psidii* soil inoculations on the growth of guava plants (cv. Fan Retief), and soil spiral nematode population

	Elapsed time period from the commencement of soil inoculations (months).					
Treatments	6	12	18	24	30	36
1, F/N						
Nematode counts[x]	<500	<500	<500	1100	<500	<500
Mean Plant height[y]		1.89b		2.65c		3.5b
2, F/N/Np						
Nematode counts[x]	<500	<500	<500	<500	<500	<500
Mean Plant height[y]		1.71b		1.66b		na
3, Np						
Nematode counts[x]	3500*	3500*	5500	5000*	6500*	4200*
Mean Plant height[y]		0.92a		1.33ab		na
4, F						
Nematode counts[x]	800	<500	<500	<500	900	700
Mean Plant height[y]		1.6 b		2.8 c		3.3b
5, Control						
Nematode counts[x]	3800*	4200*	8600*	6700*	7700*	8600*
Mean Plant height[y]		0.75a		1.18a		1.4a

[x] Soil levels of the Spiral nematode. [y] Mean plant height (m) was assessed by measuring the tree height from the soil to the highest branch.
Mean plant height figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Five replicates per treatment.
* Significantly different from the other soil nematode values in the same column according to the Waller-Duncan *k*-ratio *t* test (P=0.01).
F = fumigation; N = nematicides; Np= *N. psidii* soil inoculations; na = all plants dead due to GWD. Orchard 5(a).

As in Orchard 2, nematode levels were dramatically reduced by the chemical treatments. GWD was not observed in any of the treatments for the first year following planting. Where *N. psidii* inoculum was applied, GWD symptoms were detected in all treatment replicates by Month 30. However, where *N. psidii* inoculum was applied after fumigation, there was a six month delay in the onset of GWD symptoms (Table 10.4).

Table 10.4. The effect of pre plant fumigants, chemical nematicides and *N. psidii* soil inoculations on the progression of GWD in newly planted guava plants (cv. Fan Retief)

Elapsed time period from the commencement of soil inoculations (months).

Treatments	6	12	18	24	30	36	42	48
1, F/N GWD incidence[x]	0	0	0	0 a	0 a	60 a	60 a	60 a
2, F/N/Np GWD incidence[x]	0	0	0	40b	100c	100b	100b	100b
3, Np GWD incidence[x]	0	0	60	80c	100c	100b	100b	100b
4, F GWD incidence[x]	0	0	0	0 a	0 a	40 a	40 a	70 a
5, Control GWD incidence[x]	0	0	0	0 a	40 b	40 a	100b	100b

[x] GWD was assessed by determining if the disease was present (+ or -) in any of the replicates (expressed as a percentage) at each assessment interval. F= fumigation; N= nematicides; Np= *N. psidii* soil inoculations. Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05). Statistical analyses were not performed on values prior to the 24 month assessment date as GWD had only appeared in Treatment 3, Orchard 5(a).

Overall, treatments comprising of fumigation (without *N. psidii* inoculation) remained free of GWD for the longest period (30 months). Combined fumigation and nematicide applications slightly delayed the onset of GWD. Some fumigated replicates in these treatments remained free of GWD for a further two years (Table 10.4). However, all trees eventually succumbed at approximately 55 months from planting (data not shown).

10.4.2 Nematode interactions with *N. psidii*

Soil samples taken from guava production regions in the eastern and north eastern areas of South Africa, Swaziland and Mozambique indicated a broad distribution of the spiral nematode (Fig. 10.1). Some of these samples yielded high levels of the spiral nematode with relatively low or zero levels of other phytophagous nematode genera. Microscopic observations as to the effect of the surface sterilization treatment on the nematodes suggested that the treatment had little effect on their mobility. Greenhouse plants artificially infested with the spiral nematode exhibited severe growth retardation and significantly lower dry shoot mass production (Fig 10.2). Spiral nematode infested guava plants produced only 17 % of the dry shoot mass compared to the uninfested plants (Table 10.1). Treatments infested with the spiral nematode that were subsequently exposed to a soil drench of a conidial and mycelial mixture of *N. psidii* succumbed to GWD but at a significantly lower rate of tree mortality at 30, 60 and 90 days post inoculation compared to the fungal inoculations alone. At 120 and 150 days post inoculation there was no difference in tree mortality rates between the two treatments (Table 10.1). Four months after inoculation all plants, were dead in both the spiral nematode plus *N. psidii* treatment plots, as well as the *N. psidii* alone plots

10.4.3 Influence of the Salagena bark borer (SBB) on GWD.

Insecticidal sprays applied to the designated trees utilized in these specific trials, remained free of the bark borer until they were artificially infested. No adverse effects on the SBB larvae used to artificially infest the guava trees were observed from the chemical pre-treatment applications (pre deployment orchard insecticide sprays that were inactivated prior to insect

placement by alkaline inner canopy branch sprays).

SBB's were found to be widely distributed in the sub-tropical regions of Southern Africa, on various tree crops including pecan and guava (Fig. 10.5).
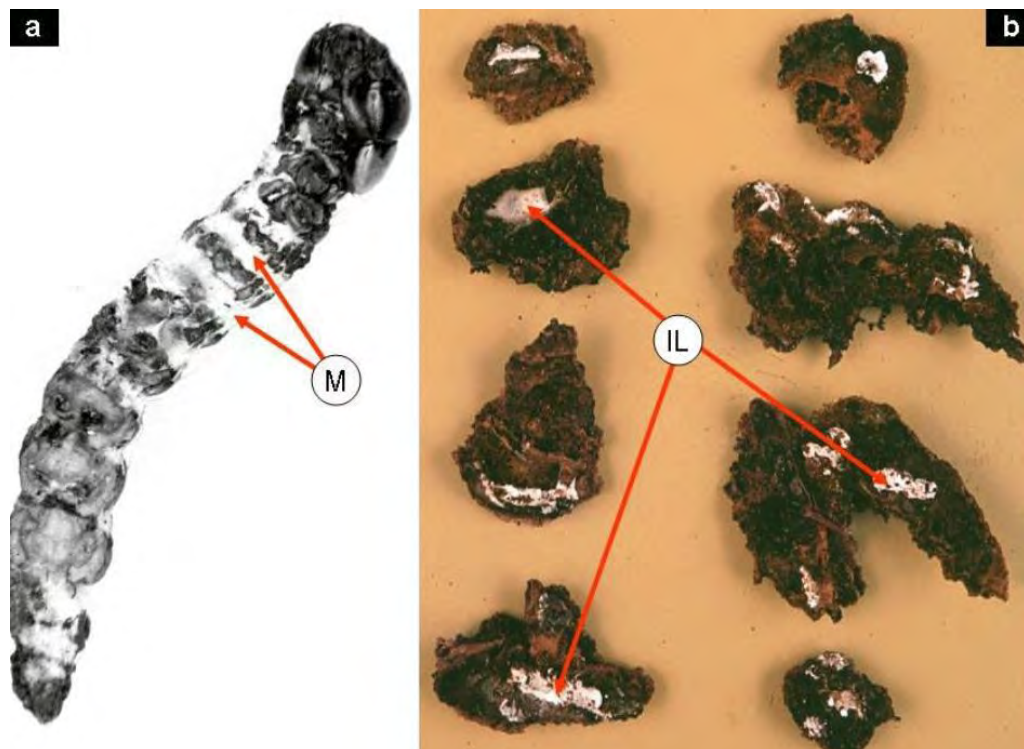
Fig. 10.5. Guava bark borer cadavers infected with the entomopathogenic fungus *Beauveria bassiana*.
a, recently deceased mature larval stage of *Salagena* sp killed by *B. bassiana*, extracted from guava wood as shown in Fig. 10.6. Note the mycelia (M) between segments.
b, *B. bassiana* infected *Salagena* sp. larvae in pecan tree bark cavities. Note the white mycelia emanating from infected larvae (IL) and permeating the chamber. Generally the larvae were found in the scaffold branches or under ant bands (Fig. 10.6). Infestation levels tended to be greater and bark damage higher where ant bands were present.
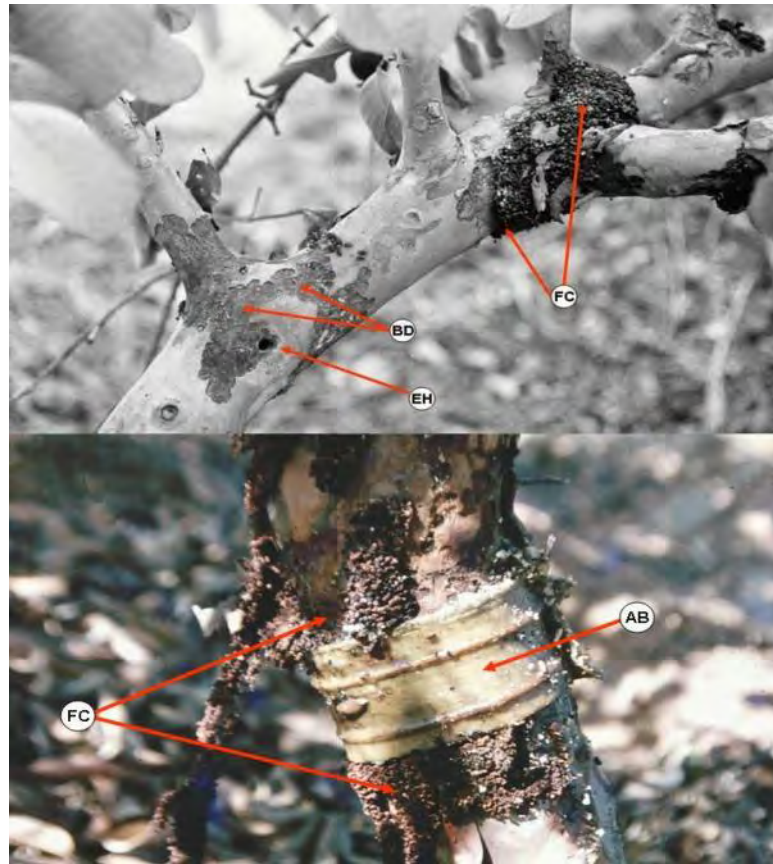
Fig. 10.6. Guava bark borer (*Salagena* sp.) damage to a field guava tree.
EH = entry hole. BD = bark damage. FC = frass covering. AB = ant barrier.
Note the bark borer frass incidence above and below the ant barriers.

Nightly collections yielded sufficient larva to conduct experiments. Preliminary tests on the larva to determine if the sterilization procedure was harmful to the insects indicated that the 20 second immersion treatment in hibitane diacetate did not negatively affect the animals in terms of mobility. The first attempts at establishing the insects during the late afternoon resulted in high mortalities due to desiccation. Subsequent insect placement was conducted during the evening and insect survival was greater than 80 %.

In infested orchards during the late autumn and early winter periods, trees at the early stages of GWD where frequently also infested with the bark borer. Attempts at recovery of *N. psidii*

from these trees were generally successful (Fig. 10.7).

Fig. 10.7. Field guava bark borer canopy damage and *N. psidii* isolation recovery.
a, branch exhibiting branch dieback symptoms.
b, major tree canopy symptoms.
c, xylem tissue isolations from bark borer infested branches onto PDA from asymptomatic field
trees in a GWD infected orchard.
1 = *N. psidii* recovery from a heavily SBB infested guava.
2 = negative *N. psidii* recovery from a lightly SBB infested guava.
CW = canopy wilt. FC = frass covering.

The SBB significantly increased inoculation success with *N. psidii*, whether placed before or

after inoculation (Table 10.5).

Table 10.5. The influence of the *Salagena* sp. bark borer on the development of GWD in field guavas (cv. Fan Retief)

	Percentage no. of plants showing GWD symptoms				
	(days from inoculation)				
	30	60	90	120	150
Experiment 1 (Np then SPP trunk application)[x]					
Np+ SPP (+ 1 hr)	0a	12.5b	12.5bc	62.5c	100 d
Np+ SPP (+ 7 days)	0a	12.5b	25 b	12.5b	62.5c
Np+ SPP (+30 days)	0a	0 a	0 a	0 a	12.5b
Np alone	0a	0 a	0 a	0 a	0 a
Experiment 2 SPP then Np trunk application)[y]					
SPP +Np (+ 1 hour)	0b	0 a	75c	75 c	100 b
SPP +Np (+7 days)	0a	25b	75c	100 d	100 b
SPP +Np (+30 days)	0a	0 a	25b	50 b	100 b
SPP alone	0a	0 a	0 a	12.5 a	12.5a

[x]*N. psidii* initially applied to the bark followed by application of *Salagena* sp.
[y] *Salagena sp* initially applied, followed by *N psidii* application to the bark.
In each experiment, figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).
Eight single tree replicates per treatment.
SPP= *Salagena* bark borer, Np = *N. psidii*.

The application of the bark borer prior to the application of *N. psidii* usually resulted in a higher levels of GWD development (particularly when the insect was actively feeding) than when the fungus was applied first. As the larvae are naturally active for at least 2-3 weeks, the application of *N. psidii* any time during this active wood boring (and hence wounding) period would be expected to produce similar levels of infection, which was in fact what was recorded in this experiment (Table 10.5, Experiment 2). There were no differences in the temporal GWD symptom expression at the 150 day post deployment assessment, between *N. psidii* being applied 1 hour or 7 days after SBB was deployed. However, when *N. psidii* was applied 30 days after the insect was positioned, GWD was less evident and developed more slowly, based on the 90 and 120 day assessment periods. This may have been due to reduced insect feeding (and hence wounding) at this time period, as the larvae would after 30 days post deployment be approaching or would have reached the pupation stages of their life cycle.

An anomaly in these data (Table 10.5, Experiment 1), in terms of GWD expression, occurred with the fungal spray application treatment that preceded the bark borer application by seven days. At the 90 day assessment of this treatment, 25% of the treatment trees were assessed as exhibiting GWD symptoms. However by 150 days only 12.5% of trees exhibited GWD symptoms. This is likely the result of an initial misdiagnosis of the foliar canopy symptoms of GWD and SBB, which in some circumstance, may be similar (Fig. 10.7 and 10.8).
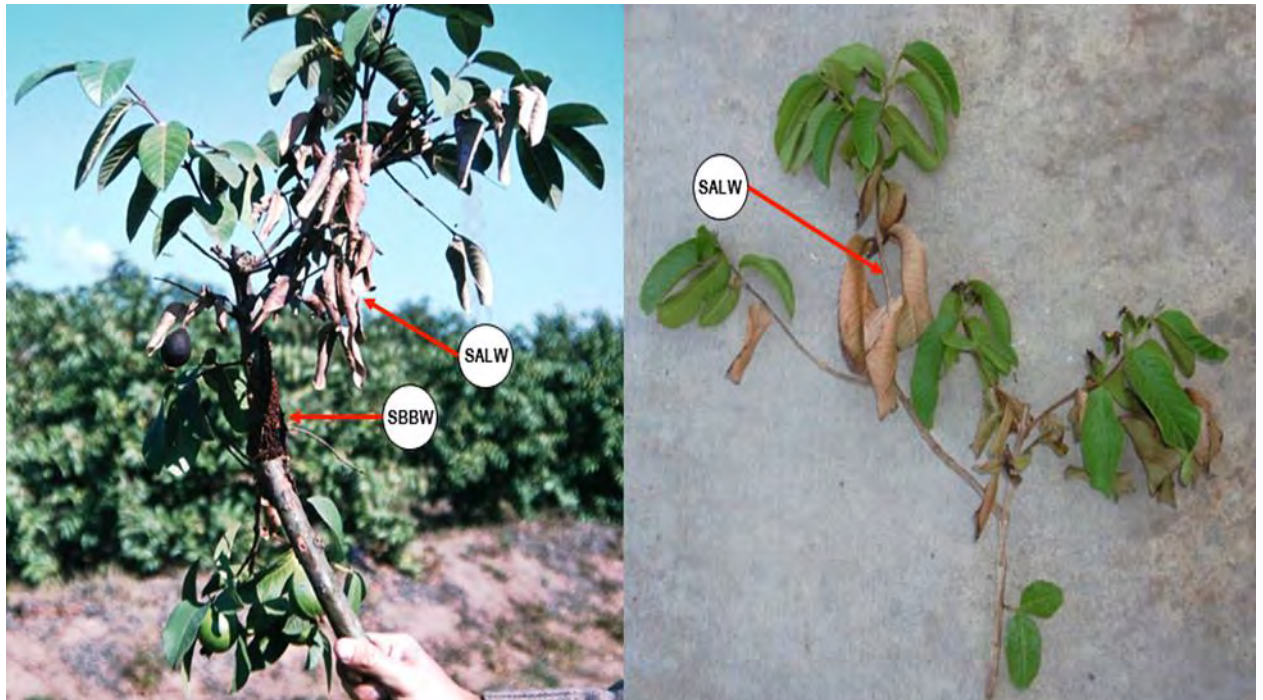
Fig. 10.8. Comparison of *Salagena* bark borer (left) induced branch wilting as compared to that induced by GWD (right).
Note, both maladies induce a sub-apical leaf wilting (SALW) that are similar in appearance, with the exception that SBB produces characteristic, frass covered, bark and wood wounding as a consequence of insect feeding (SBBW).

When the fungus was applied prior to insect deployment, GWD expression occurred but with a lower intensity and a delay in symptom expression than when *N. psidii* was applied after insect deployment. This was particularly evident where SBB was applied 30 days after the application of *N. psidii*. Conidia applied to the unwounded branch surfaces may have been subject to climatic factors leading to their removal (wind or rain) or loss in viability (solar ultraviolet radiation). Conversely, application of *N. psidii* 30 days after the SBB placement resulted in greater GWD development over the same time period.

10.4.4 Biocontrol of Salagena Bark Borers

Application of *B. bassiana* conidia induced significant mortality in SBB larvae within 30 days of application (Fig. 10.5 and Table 10.6).

Table 10.6. The effect of biological or chemical control of the *Salagena* sp. bark borer infestations on the incidence of GWD in field trees

Treatment[y]	Percentage of treatment trees exhibiting GWD symptoms over six months[x].		
	90 days	180 days	% of trees with 1 or more larvae[z]
1. Alternating monthly cover spray program of malathion 62.5g a.i./ 100 L) or cypermethrin (5g a.i/100 L	25ab	70a	7 a
2. *Beauveria bassiana* conidial spray at +/- 5 x 10^5 spores per ml, applied monthly.	15a	85a	20b
3. Control	40b	75a	73c

[x] After commencement of the bark borer control program (approximately three months prior to natural bark borer infestation periods).
[y] Treatments were commenced at least 3 mo. prior to the appearance of bark borer damage (May-June).
[z] Treatment trees were assessed for the level of SBB infestation and trees with one or more larva were counted.
Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).
Fifteen single tree replicates per treatment.

High levels of germinating conidia were observed on the Petri plate media that were sprayed in the orchard during the spray application. *B. bassiana* was successfully re-isolated from

collected moribund and dead SBB larvae in all the treatments were it was applied. Both chemical and biological treatments reduced levels of the bark borer in *N. psidii* infected orchards. GWD incidence at the 90 day assessment was reduced by the *B. bassiana* (and numerically by the chemical treatments) compared to the Control. The reduction in GWD incidence by the chemical and biological treatments did not persist and by 180 days post treatment, GWD was at similar levels across both treatments and the Control. Removal of frass around bark borer tunnels has no impact on larval infectivity of *B. bassiana.*

10.5 DISCUSSION

Nematodes were found ubiquitously in guava orchards, with the spiral nematode being the most commonly detected pathogenic nematode (Fig. 10.1), a situation similar to that occurring in Taiwan (Anonymous, 1984). In greenhouse studies at soil populations comparable to the field, the spiral nematode reduced the growth of guava plants by 83% over 150 days (Table 10.1 and Fig. 10.2). Inoculations of *N. psidii*, combined with the spiral nematode, did not accelerate the development of GWD in greenhouse studies (Table 10.1).

A number of nematodes species are found in the rhizosphere of cultivated guavas in Southern Africa (Fig. 10.1). The spiral nematode is most commonly associated with guavas and can induce significant negative impacts on roots and overall plant growth if their population is left unabated (Figs. 10.3 and 10.4). The spiral nematode is also found in association with other nematodes such as the root knot nematode (particularly in the warmer production regions), and when present together, these nematodes have the ability to induce significant damage to guava

roots (Willers and Welgemoed, 1993). Subsequent studies have identified the root knot

nematode associated with guava in the subtropical Mpumalanga lowlands of South Africa as

Meloidogyne mayaguensis Rammah and Hirschmann, 1988 (Willers, 1997).

Soil applied nematicides alone or in combination with pre-plant fumigation had a significant

effect in decreasing soil spiral nematode counts and positively impacting upon plant growth

(Tables 10.2 and 10.3). This was consistent with previous studies where populations of the

spiral nematode on guava growth indicated a significant negative effect of these parasites on

newly planted guava trees (Willers and Grech, 1986). However in these trials where

nematicides were applied alone, GWD was not recorded for the entire duration of the trial and

hence no conclusions could be drawn as to the effect of these chemicals on the disease (Table

10.2).

Test Orchard 2 (Chapter 5, Table 5.1) was also found to have plant growth limiting soil levels

of the spiral nematode. However, the combination of fumigation and post-plant nematicides

significantly reduced soil population levels in Orchard 5(a) as seen in Table 10.3 and resulted

increased plant growth. Control trees were found across all plant height assessment periods, to

be approximately 40% of the size of the treated trees (Fig 10.4 and Table 10.3). These findings

confirmed the results of earlier greenhouse experiments that indicated the spiral nematode can

induce severe dwarfing effects on guava growth (Figs. 10.2 and 10.4).

Over the course of the experiment in Orchard 5(a), *N. psidii* did not spread from surrounding

orchards to the fenced area, and as such, it is presumed that natural infection did not impact

312

this experiment to any significant effect. Both treatments where *N. psidii* was inoculated into the rhizosphere with or without fumigation, resulted in the most rapid detection of GWD (Table 10.4). However, in previous *N. psidii* rhizosphere inoculations of guavas, where the pathogen was introduced and mechanical wounding was undertaken, GWD symptoms were seen to develop more rapidly at both field inoculation sites (Chapter 8, Table 8.2). Although these field studies only indicated a slight reduction as to the onset of GWD symptoms where nematodes were controlled, the role of nematodes in this disease malady warrants further study. Fumigation consistently resulted in delayed detection of GWD compared to the Control. In fact these treatments provided the longest protection from GWD. Treatments were assessed in terms of the appearance of symptoms of GWD and not plant mortality (although plant death always followed symptom expression after one to two months depending on the season). Even after 55 months, some fumigated plots still had trees that were disease free (data not presented). In this particular trial, drip irrigation was postulated to mitigate against irrigation dissemination of *N. psidii* as discussed in Chapter 5. However, as fumigation was not applied to the whole experimental area, but was applied only to small plots within the fenced enclosure, migration of the pathogen into fumigated plots from surrounding unfumigated soil and from dead infected root matter were possibilities, particularly during the rainy season (summer). However, the impact of this seemed to be low because the Control trees were asymptomatic for up to twenty four months. These data are encouraging in developing treatment programs for GWD in new orchards. In these experiments, pruning was not conducted due to the possible impact on these experiments. However, commercially this would be unacceptable and therefore any rhizosphere treatment program for GWD should be coupled with pruning wound protection. It is possible that the many biotic and abiotic wounding factors present in the rhizosphere could

have masked the effect of the nematodes on GWD. At higher nematode populations in the field, it may be possible to demonstrate that the spiral nematode may expedite the development of GWD. Recently it has been demonstrated that mixed populations of the spiral and lance nematodes (*Hoplolianus* spp.) increased the *Fusarium oxysporum* induced guava wilt reported from India and Pakistan (Hamiduzzaman *et al*., 1997). In these trials, the use of fumigation with or without chemical nematode management, reduced the incidence and/or disease progress of GWD (Table 10.4). Although not tested, it is possible that the other members of the plant pathogenic nematode genera found on guava roots may also exacerbate GWD, as reflected by the many interactions reported to occur between plant pathogenic nematodes and fungal plant pathogens (Neal, 1954; Mace *et al*., 1981). The root knot nematode, well known for its many associations with plant pathogenic fungi (Wilheim and Paulus, 1980; Cohn and Duncan, 1981; Greipsson and El-Mayas, 2002), is reported to be the most damaging nematode in guava cultivation worldwide in relation to its population on roots (Fernandez *et al*., 1986, 1987a, 1987b, 1987c; Moura and Moura, 1989; Carillo *et al*., 1990). Fortunately this nematode is not common in guava cultivation in Southern Africa, although when present can induce severe galling (Fig. 10.3). In those parts of the world where the root knot nematode is limiting to production, control measures are currently reliant on soil applied organophosphate or carbamate nematicide applications (Carillo *et al*., 1990). Success with biological control of certain nematode species has been reported with entomopathogenic fungi (Cabrera, *et al*., 1987).

In the sub-tropical regions of South Africa, two species of the carpenter moth (*S. obsolescens* Hampson and *S. transversia* Walker) belonging to the genus *Salagena* are known to feed on a

number of trees including guava (De Villiers, 2001). The SBB is a regular seasonal pest of guava in the sub-tropical northern and north eastern guava production regions of Southern Africa (De Villiers and Joubert, 2004). The larval stages of this carpenter moth burrow into the woody tissue of trees and emerge during the night to feed on the bark of the host tree. The moth larvae are found on a broad range of hardwood trees such as litchi, pecans, avocado, macadamia and many indigenous non-crop tree species. Bark boring insects (including members of the *Metarbelidae* family) are commonly associated with guava cultivation in many parts of the world (Holloway, 1986; Sharma and Kumar, 1986; Sandhu *et al.*, 1987; Singh, 1988; Latis, 1990; Shevale, 1991).The insect was found ubiquitously in guava orchards located in the sub-tropical regions of Southern Africa during late autumn and early winter (Fig. 10.6), inflicting significant damage to the bark tissues of guava trees throughout the canopy. Interestingly, damage was found to be exacerbated by the use of ant bands, used for the control of mealy bug, by disrupting ant/mealybug mutualism (Samways, 1982). Guava orchards infected with *N. psidii* were similarly found to be infested with the bark borer during the same period. The SBB would induce sectorial wilting in the bark canopy, the symptomology of which could be confused with GWD (Figs. 10.7 and Fig. 10.8). In fact, in many commercial orchards in GWD afflicted regions of South Africa, trees were infested and infected concurrently (Fig. 10.7). Tree branches infested and damaged by the bark borer were frequently found (in *N. psidii* infected orchards) to concomitantly be infected with *N. psidii*.

Pre insect deployment chemical sprays that were applied several months prior to the experimental commencement, designed to maintain designated trees free of the SBB, were successful and did not induce mortality in the applied SBB larvae.

Wounding of guava wood and bark tissues by the SBB increased the development of GWD in field trees (Table 10.5), independent of the order that the insect or the fungus was applied in. However, differences in GWD mortality rates between treatments were apparent. Where *N. psidii* was initially applied, application of the insect within one hour after the inoculum was deployed, resulted in the highest level of incidence of GWD (Table 10.5, Experiment 1). Deployment of the larvae at 7 and 30 days after *N. psidii* was applied, increased the level of GWD in treated plants (Table 10.5), but at lower level than when the larvae were applied at 1 hour after the pathogen. In the 30 day post application of SBB larvae, the one tree that was symptomatic may have been naturally infected due to the delay in the onset of GWD symptoms, which only manifested at 150 days (Table 10.5, Experiment 1).

It is interesting to note the results of the 90 day assessment as shown in Table 10.5 (Experiment 1). In the treatment where the bark borer larvae were deployed 7 days after *N. psidii*, GWD was recorded in 25 % of the treatment replicates. However, by the 120 day assessment, this level was 12.5 % and remained so until the end of the experiment (150 days). It is postulated that this finding was the result of an initial misdiagnosis by the author as to the causal agent based on the symptom expression. As previously noted, GWD and the SBB can induce similar symptoms in the guava canopy (Figs. 10.7 and 10.8).

Where the SBB larvae were applied prior to *N. psidii*, all treatments separated significantly from the insect only treatment at the 150 day assessment interval (Table 10.5, Experiment 2). One tree in the SBB only treatment developed GWD, probably through natural infection,

whereas all the other trees in the treatment remained GWD free. Although the insect larva usually excavates only one hole into the wood, it is likely that the continued wounding to the bark and wood that the SBB inflicted on the tree, maintained fresh wounds on the guava tree canopy for the duration of the insect's larval stage and hence created potential infection courts for *N. psidii*. It is uncertain as to whether the covering of frass, held together with silk that the insect constructs over its burrow and feeding areas, is an impediment to successful infection by *N. psidii* spores. Earlier experiments in this study (Grech, unpublished) have shown that 'fresh' wounds are more receptive to *N. psidii* invasion than 'aged' wounds. The finding in these trials that inoculum application after insect colonization resulted in generally a higher degree of GWD development than when inoculum was applied prior to the insect was established, is interesting but is unlikely to be of any significance in terms of the field management of GWD, where the avoidance of wounding agents in the canopy is critical.

The SBB in these studies was found in many trees present in areas where guava are grown in the northern and north eastern areas of Southern Africa. Pecan trees also are heavily affected by the SBB in South Africa. Field surveys of pecan trees resulted in the collection of SBB cadavers (Fig. 10.5) infected with a fungus that was subsequently identified by the author as *Beauveria bassiana* (Bals.) Vuill., a species well documented to be entomopathogenic on Lepidopteran insects (Legaspi *et al.*, 2000). The fungus was successfully cultured and field applied to guava trees. The fungus was successfully re-isolated from SBB cadavers. *B. bassiana* is ingested by the insect as it consumes guava bark treated with spores of the fungus (Huang *et al.*, 1990). After ingestion the fungus would systemically invades the insect's haemolymph, leading to host death, after which it would erupt out of the body and sporulate

profusely on the insect cadaver as can be seen in Fig. 10.5, which was extracted from a guava scaffold branch. This is the first report of this entomopathogenic fungus being isolated from bark boring *Salagena* spp infesting guava in Southern Africa.

Biological control of the SBB in the field reduced the percentage incidence of GWD during the first 90 days (Table 10.6), as compared to the Controls, whereas the chemical treatments resulted in a similar percentage incidence of GWD during the first 90 days as the biological control treatment (but not significantly different from the Control). At 180 days post tree inoculation with *N. psidii* there were no significant differences between the trees receiving the bark borer control programs and the untreated trees. Unfortunately, some natural infestation by the SBB occurred in the experimental blocks, with the result that some treatment trees had multiple infestations (Fig. 14.6). In future experiments, an expanded area as well as the number of single tree replicates that receive intensive pre-treatment insecticide sprays may allow for less natural SBB infestation of experimental trees.

The wood boring activities of the bark borer in guava orchards is restricted to the winter months, coinciding with the fruit harvest periods, and therefore increased orchard traffic. During these periods it has been shown that the occurrence of in-orchard and between-orchard spread of *N. psidii* is high (Chapter 5), so bark borer control would seem logical, even though these field control experiments were not conclusive over the entire period of experimentation. Control of guava bark boring insects in other parts of the world include chemical (Sandu *et al.*, 1987; Singh, 1988) and biological methods (Fasih and Srivastava, 1988; Huang *et al.*, 1990). *B. bassiana* has previously been isolated from beneath the bark on larvae associated with Dutch

elm disease (Doberski and Tribe, 1980). Because the SBB induces significant damage to guavas in Southern Africa, extensively wounding wood and bark tissues of the tree primarily during the winter months (Figs. 10.6 and 10.7), this pest also has the potential to exacerbate GWD levels in commercial orchards and thus should be managed.

In its native habitat of the south eastern regions of the United States, the common persimmon (*Diospyros virginiana*) is attacked by several bark and wood boring insects (Halls, 1981; Holdeman, 1998): such as wood and bark borer larvae from the clear winged moth family, *Sesiidae*; the persimmon root borer (*Sannina uroceriformis* Walker) also from the *Sesiidae* (persimmon is the only know host for this species); the bark and phloem beetle and larva borer (*Agrilus fuscipennis* Gory*),* persimmon being the only know host for the larvae and the persimmon bark borer (also known as the quince moth), *Euzophera batangensis* Caradja, which is an imported pest from east Asia.

Both moths, as well as the beetle, have been implicated as the wounding agents for the persimmon wilt pathogen, *Nalanthamala diospyri* (Crandall) Schroers and Wingf., comb. nov. persimmon wilt was first identified in the south eastern United States (Tennessee) in the early 1930's (Fletcher and Gould, 1942), and by the end of the decade, it had rapidly spread throughout Mississippi, Alabama, Georgia, South Carolina, North Carolina, Texas and Florida (an area approximately twice the land area of South Africa). This disease accounts for the current lack of commercial American persimmon cultivation in the south eastern United States. Previously the persimmon wilt fungus was known as *Cephalosporium diospyri* (Crandall 1945), was reclassified by Gams (1971) as *Acremonium diospyri* and was most recently

reclassified as *Nalanthamala diospyri* (Schroers *et al*., 2005). The etiology of this disease closely resembles GWD in many aspects of its life cycle, including the following: it is a wound pathogen; bark boring insects provide bark and wood wound entry points; pruning (not widely practiced in persimmons grown in the Eastern USA) also provides wound entry; the causal fungus produces two spore types and sporulates profusely under the bark; the pathogen rapidly spreads by root to root contact and root anastomosis. Further, *N. psidii* is postulated as having originated in Asia where currently it has a significant distribution (Schroers *et al.,* 2005); *N. diospyri* is also thought to have originated in Asia and to have been introduced unknowingly on Asian persimmon variety introductions of *D. kaki* and *D. lotus* (Halls, 1981).

Descriptions of the symptomology according to Crandall (1939 and 1945), Wilson (1963), McRitchie (1979) and Holdeman (1998), of persimmon wilt, closely parallels that of GWD symptomatology, particularly in terms of the rapidity of death (two months), canopy symptoms (rapid wilting and defoliation), spreading by root grafts, tissue discoloration (black streaks in the wood) and erumpent sporulation (pink spore masses of the fungus produced beneath the bark). The impact of persimmon wilt in the first half of the 20th century in the south eastern USA was clearly described by McRitchie (1979) in terms of the rapidity and virulence of the pathogen as quoted from his paper, "*The fungus, Cephalosporium diospyri, virtually eliminated persimmon from one of its main habitats, the central basin of Tennessee, and has been found south to Florida and west to Oklahoma. The pathogen is so efficient that it was once proposed as a biological control agent to eliminate unwanted native persimmons*". The description of the persimmon epidemic in the USA closely resembles the decimation of the guava industry in the Province of Mpumalanga as a result of GWD. Currently no acceptable level of commercial

genetic tolerance to persimmon wilt has been identified in *D. virginiana* because the industry is highly reliant on the horizontally resistant Asian species (*Diospyros kaki* and *D. lotus).* Certain sycamore species are also susceptible to the persimmon wilt fungus, *N. diospyri.* (Leininger *et al*., 1999). Interestingly, bark and wood boring insect (primarily Lepidopteran) are also associated with the wilting syndromes associated with these tree genera. Control of these insects is advocated in managing these wilting maladies (McRitchie, 1979; Holdeman, 1998). In Florida, the twig girdling beetles have been implicated in transmitting *N. diospyri* between trees (Burns and Honkala, 1990; Mizell, 1998; Frank and Mizell, 2009), as once the beetle larva pupate, the adults emerge and take flight to another tree, where the adults will feed on persimmon bark. In Southern Africa, the Lepidopteran *Salagena* bark borers do not as adults feed on bark or wood. They mate and lay eggs on susceptible hosts, which hatch and begin feeding on bark and wood. Several Coleopteran beetles have been observed infesting GWD wilted branches as well as dead trees, both of which have had active sporulation (Grech, unpublished). The role of these insects in the etiology of GWD requires further investigation. However, in the case of the closely related pathogen *N. diospyri*, the role of wood boring insects is clearly implicated and control of these animals are recommended as part of an overall management program for persimmon wilt (Crandall and Baker, 1950).

Both nematodes and particularly the *Salagena* carpenter moth larva exacerbated GWD and as such further expanded studies specifically investigating the impact of these animals on GWD and the potential for reducing the infectivity of *N. psidii* by greater management of these invertebrates.

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324

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CHAPTER 11: LABORATORY SENSITIVITY OF *NALANTHAMALA PSIDII* TO METHYL BROMIDE, AND FIELD EVALUATION OF THE FUMIGANT FOR THE ERADICATION OF *NALANTHAMALA PSIDII* IN CONTAMINATED ORCHARD SITES

ABSTRACT

Unprotected inocula of *Nalanthamala psidii* (Biourge) Shroers was completely killed *in vitro* by 75g.m^{-3} of the fumigant methyl bromide (MeBr). Field fumigation under plastic tarp (which elevated soil temperatures compared to the untarped by 26°C and 20°C at depths of 100 mm and 300 mm, respectively), was successful at eliminating *N. psidii* unprotected inocula as well as infected feeder and tertiary roots. However, the pathogen was not eliminated from infected root tissues with diameters of 1 cm and above. In agreement with earlier studies, MeBr significantly reduced nematode populations in field soil and retarded the development of guava wilt disease (GWD) in new trees planted into fumigated soil, over a 36 month period. Tree mortality in the fumigated plots was reduced by over 50%. However, due to the high level of susceptibility of commercial varieties of guava, and the high dispersive potential of *N. psidii*, this level of control was inadequate. Initial deployment, in fumigated and unfumigated experimental sites, with the putatively resistant experimental guava selection, OT-1, did not result in any tree mortality as a result of GWD.

11.1   INTRODUCTION

Nalanthamala psidii (Biourge) Schroers is an aggressive wound pathogen of guava that is highly destructive to guava plantations. When present in orchards with conventional management regimes encompassing extensive wounding practices and poor orchard sanitation, this fungus will induce rapid and extensive tree death. Given that none of the current commercial guava cultivars are resistant to guava wilt disease (GWD), replanting guavas into orchard soils previously infested with *N. psidii* is not viable. Previously (Grech, unpublished), it was shown that viable infective propagules can remain in plant tissues (such as major roots and stems) for at least 12 months, whereas in soil the pathogen persistence was only 3 months. Therefore there is a need to develop a treatment to eradicate infective propagules of *N. psidii* in the soils of orchards previously infected with the pathogen. Methyl bromide has been successfully used as a soil fumigant against many plant vascular wilting pathogens (Ebben *et al*., 1983; Herbert and Marx, 1990; Gamliel *et al*., 1997) and was commonly utilized in guava nurseries (Fernandez, *et al*., 1987). The aim of this study was to evaluate methyl bromide fumigation as a method to sanitize orchards soils and eradicate *N. psidii* prior to replanting.

Methyl bromide has subsequently been banned globally (http://www.epa.gov/ozone/mbr/) in order to protect the ozone layer. However, these trials remain relevant in showing the potential of soil fumigants such as metham sodium, dazomet, methyl iodide and chlorpicrin, which remain unbanned (http://www2.epa.gov/soil-fumigants/regulatory-status-fumigants). Molar efficacy of methyl iodide has been shown previously to have comparable (and in several disease scenarios, superior) efficacy to methyl bromide as well as improved handling attributes and

unlike methyl bromide, does not damage the stratospheric ozone layer (Ohr et al., 1996).

11.2 MATERIALS AND METHODS

11.2.1 Fumigant sensitivity of *N. psidii*

Laboratory assessment of the sensitivity of *N. psidii* to methyl bromide fumigation was assessed by growing the fungus aseptically in a Czapek-Dox broth liquid culture medium, amended with wheat seeds and washed guava feeder roots that were introduced as bait for *N. psidii*. The cultures were incubated at 30°C under a 12h day/12 night lighting regime for 7 days under continuous rotary agitation (60 rotations/minute). This incubation method was shown previously to produce three spore types (Type 1, Type 2 and chlamydospores), as described in Chapter 4. The inoculum was drained of spent broth and mixed with sterilized, autoclaved potting soil in a 1:10 w/w ratio. 100 g of this mixture was placed in a cheese cloth seed bag, and the top tied closed. This mixture was placed in a sealable wide mouth fumigation jar (2 liter capacity glass canning jars) and refrigerated until used, within 2-3 hours.

Methyl bromide containing canisters (454g in each canister; Dead Sea Bromine Company, Tel Aviv, Israel) were chilled to -56°C using dry ice. At this temperature methyl bromide is a non-volatile liquid. In addition, glassware and micro-pipette tips were also chilled prior to use by placing them in an ice chest containing dry ice. The wide mouth jars were placed horizontally under a fume hood. The chilled methyl bromide was poured into a chilled beaker under a fume hood, and pipetted onto a small chilled glass evaporating dish close to the mouth of the jar. The jars were sealed and left on the laboratory bench for 1, 2 and 4 days. Each treatment was replicated four times. Methyl bromide was used at the equivalent field application rates of 50,

75 and 150 g.m^{-3}. At the end of the fumigant exposure period, the jars were opened under the fume hood and left to aerate for three hours, after which the cheese cloth seed bags were removed from the wide mouthed jars and transferred to a surface sterilized glass dish on a laminar flow bench. The bags were opened and bait samples, namely five wheat seeds plus five guava feeder root pieces, were placed on half strength acidified corn meal agar amended with 250mg.L^{-1} ampicillin (CMA$_{aa}$). Two Petri plates/replicate were incubated at 25° C under a 12h day/12 night lighting for three days after which they were inspected for the presence of *N. psidii*.

11.2.2 Field soil fumigation

Orchards 2, 4 and 5 (Chapter 5, Table 5.1) in Mpumalanga, South Africa, were selected for field soil fumigation studies. These orchards were found to have a wide range of GWD infection (4 to 28%) at the onset of these studies. These orchards also had high populations of the spiral nematode, *Helicotylenchus dihystera* (Cobb) Sher, which may enhance the movement and infectivity of *N. psidii*. Two guava varieties were used as replants (cv. Fan Retief and an experimental selection, OT-1). In Orchard 4, Fan Retief was used exclusively. Dead and diseased trees were removed and burnt to eliminate fresh inoculum emanating from moribund or dead trees. Healthy trees adjacent to or close to dead trees that were scheduled to be removed, were sprayed with glyphosate (Round Up$^®$ 360g a.i.L^{-1}, Monsanto, Bryanston, RSA) herbicide, applied as a 0.75% v/v solution of RoundUp$^®$ with 5% w/v ammonium sulfate added to acidify the solution. This solution was applied by a motorized low drift sprayer at 15 liters/tree. One week after complete collapse of the canopy and death, the trees were also

331

removed and burnt. After burning, the ashes were spread evenly across the orchard to eliminate any localized nutritional effects of the tree ashes. The plots were ripped to a depth of 1 meter along both the N-S and E-W planes, followed by deep ploughing (50 cm deep). Finally the soil was harrowed, cultivated to a fine tilth and planted to irrigated maize until the following early summer (late October/early November). After the maize harvest (where possible), the corn stover was ploughed back into the soil followed by harrowing. A pre-emergent herbicide was applied to the soil after which these sites were left undisturbed for a further 2 months after which they were prepared for soil fumigation utilizing the 'hot gas' methyl bromide injection method as described below. Planting row beds (3m wide) were prepared by a tractor drawn harrowing and bed forming device. Planting holes were marked at 2.5m spacings in the row. Depending on the site, tree rows were planted 5-6 meters apart. Polypropylene irrigation tube (13mm inner diameter) was laid out with 4 liter per hour drip irrigation emitters inserted into the line corresponding to the planting holes. The soil was irrigated to approximately 75% of field capacity, to a depth of 600 mm, and left for 2 days for soil moisture to equilibrate.

Prior to the plastic tarpaulins being deployed, primary roots of healthy guava trees (30-50 mm x 150 mm) were soaked in Czapek-Dox broth overnight, placed in autoclavable plastic bags, autoclaved and inoculated with *N. psidii*. The broth was autoclaved and inoculated with *N. psidii* and incubated in darkness at 30^OC. After 14 days the mycelia was removed and drained. The inoculated root pieces were placed in an incubator at 30° C under a 12h day/12 night lighting regime for 20 days. Color 95, TLD 18 W cool fluorescent daylight tubes (Philips Pty, Ltd, Johannesburg) were used to illuminate the cultures (placed 30cm below the lamps), emitting a nominal visible spectral intensity of approximately 2.16×10^{-3} W cm^{-2}. After 20 days,

the cultures were inspected for the presence of surface sporulation and if abundant (Figures 6.4 and 6.8, Chapter 6), were utilized. If not, then they were further incubated until an acceptable level of surface sporulation had occurred. In addition, fresh guava lateral roots (1 cm in diameter and 2 cm long) were harvested from 2 year old healthy nursery trees, washed and autoclaved. These root pieces were placed on a 6 day old actively growing culture of *N. psidii* on PDA. After 4 days of exposure to the culture, the root pieces were removed. One piece of the large primary root and three pieces of the smaller lateral roots were placed into polyester mesh bags. Ten bags were deployed per fumigation replicate. Additionally, presoaked wheat seed was placed in autoclavable plastic bags, autoclaved, inoculated with *N. psidii*, and incubated as above. The liquid cultured drained mycelium of *N. psidii* was mixed with the wheat seed and one gram of this inoculum was placed in perforated plastic capsules attached to a wooden stake (Fig. 11.1c). Ten wooden stake inoculum capsules were deployed per replicate. All the inocula were buried no further than 0.5 M from the medial axis of the plastic tarpaulin in an alternating pattern of 250mm and 500mm below the soil line, positioned in a 'zig-zag' configuration across each replicate. The soil around the inocula was compacted to the same degree as the surrounding soil and checked with a soil penetrometer to ensure uniformity. Prior to placement, a small sample of inoculum was removed from 3 bags (root pieces) and three capsules (wheat seeds and mycelia), from each replicate and plated onto CMA_{aa}, incubated at 25°C°C and checked for growth of *N. psidii* after 2 days to check on the viability of the fungus.

Soil temperatures were recorded in Orchard 4 in both the covered fumigation plots as well as in the uncovered control plots at 100mm and 300 mm by inserting a thermocouple attached to a mechanical recording thermograph (K. Fischer, GmbH, Drebach, Germany) into the soil. In

the covered plots, the probes were inserted through the plastic tarpaulin and the insertion hole sealed with tape). Soil temperatures were recorded during the soil fumigation process (7 days) in one replicate of the fumigated treatments and one replicate of the unfumigated control. Treatments were arranged into 5 replicates, each of 12 trees.

Methyl bromide was applied at $75g/M^2$. Application of methyl bromide (Metabrom, Landkem, Johannesburg) was by the 'hot gas'' method (Anonymous, 1982). The fumigant was heated to 65°C°C by passage through a hot water heat exchanger (Landkem, Johannesburg) and introduced under pressure (2.25-2.5 kg cm^2) underneath the plastic tarpaulin through perforated polyethylene `lay-flat' tubing. The perforations in this tubing were calibrated to ensure uniform gas distribution underneath the plastic tarpaulin. After fumigation, the plastic tarpaulins remained in place for 7 days, after which they were removed and the soil was allowed to ventilate. After one day all the inocula was removed from the soil. The contents of the inoculum capsules were removed and five wheat seeds and five small mycelia/soil mix samples (+/- 0.05g, equivalent to a pin head) were plated onto CMA$_{aa}$. The Petri plates (two per inoculum capsule) were incubated at 25°C°C in darkness for three days after which they were checked for growth of *N. psidii*. The root piece inocula were removed and a middle transverse disc excised from each root piece. A 10 mm strip across the larger root section diameter was prepared and pared into a center section and two outer pieces. These three pieces from the larger root and three center sections from the smaller root inocula were plated onto CMA$_{aa}$ and incubated as above (one Petri plate per root inoculum bag).

Clonal, 2 year old guava trees were planted at a 2 to 2.5 m (depending on the site) spacing in the row and 5-6 m between the rows. The treatments located in Orchards 2 and 5 were planted

in an alternating sequence of two varieties (cv. Fan Retief and the experimental selection, OT-1). In each replicate there were 12 trees in total, 6 trees of each variety. In Orchard 4, only the Fan Retief variety was utilized. Trees were replanted at an orchard tree density corresponding to the pre-removal orchard density (approximately 700-1000 trees.ha^{-1}). Trees were planted where possible in a north-south orientation. Plant stations were approximately equidistant between the positions of the tree rows of the previous orchard, prior to tree removal.

All replicate plots in Orchard 4 were split across the row and half of the trees received a quarterly full cover fungicide spray of a tank mixture of 100g benomyl (Benlate®, 50 WP, Du Pont, Johannesburg), 200g mancozeb (Dithane 80 WP, Rohm and Haas, Johannesburg) and 100g of malathion (Malasol®, Agrihold, Halfway House, South Africa), all diluted in 100 liters of water. All plants were maintained under normal cultural management, except for the absence of annual pruning, which was not conducted for the first two years of the experiment, or hand weeding. Chemical weed control was the only weed control method used in these trials, in order to avoid damage to tree roots. All three sites were monitored at least once every four months over a three year period. Replicates were scored positive or negative for the presence of GWD. The number of tree deaths per plot at all sites was assessed at 36 months. Nematode samples were collected annually, in summer, at all three sites by taking four soil sub-samples per each replicate (200 grams soil at 200 mm depth in the tree root zone) of each plot and pooling the sub-samples into a single composite sample. This was analyzed for phytopathogenic nematodes using standard soil sugar flotation extraction methodologies as described in Chapter 14 (Escobar and Rodriquez, 1980; Hooper et al., 2005).

11.3   DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, means were separated using the Waller-Duncan *k*-ratio *t* test (Little and Hills 1990).

11.4   RESULTS

11.4.1 Fumigant sensitivity of *N. psidii*

Sensitivity screening of *N. psidii* to methyl bromide *in vitro* indicated that the fungus was completely eradicated by the fumigant at the $50g.m^{-3}$ dosage rate at a minimum exposure period of 2 days (Table 11.1). Higher dosages of methyl bromide, at all duration periods tested, also completely killed *N. psidii* (Table 11.1).

Table 11.1. Recovery of *N. psidii* from infected autoclaved wheat seed and guava feeder roots following fumigation of orchard soil treated with methyl bromide for different exposure periods, in a laboratory trial

MeBr Concentration	Mean percentage recovery of *N. psidii* at different time intervals		
	Exposure Period (days)		
	1	2	4
0 g/m^3	100c	100b	100 b
50 g/m^3	50 b	0 a	0 a
75 g/m^3	0 a	0 a	0 a
150 g/m^3	0 a	0 a	0 a

4 replicates per treatment.
Values represent the mean re-isolation incidence of *N. psidii* from all four replicate assessed as
'+' or '-' and expressed as a percentage of the total number of replicates.
Means followed by the same letter in each column do not differ from one another according to
the Waller-Duncan *k*-ratio *t* test (P=0.05).

11.4.2 Field soil fumigation

Use of plastic tarpaulin covering of the fumigation beds (Figs. 11.1 and 11.2) resulted in

substantially elevated soil temperatures during late October/early November at both the 100

mm and 300 mm soil depths (Fig. 11.3). In the tarpaulin covered plots soil temperatures reached

a maximum of 49°C°C at 100 mm depth and 41°C°C at 300 mm depth. This compares to

temperature maxima of 24° C and 18° C in the non-covered plots.
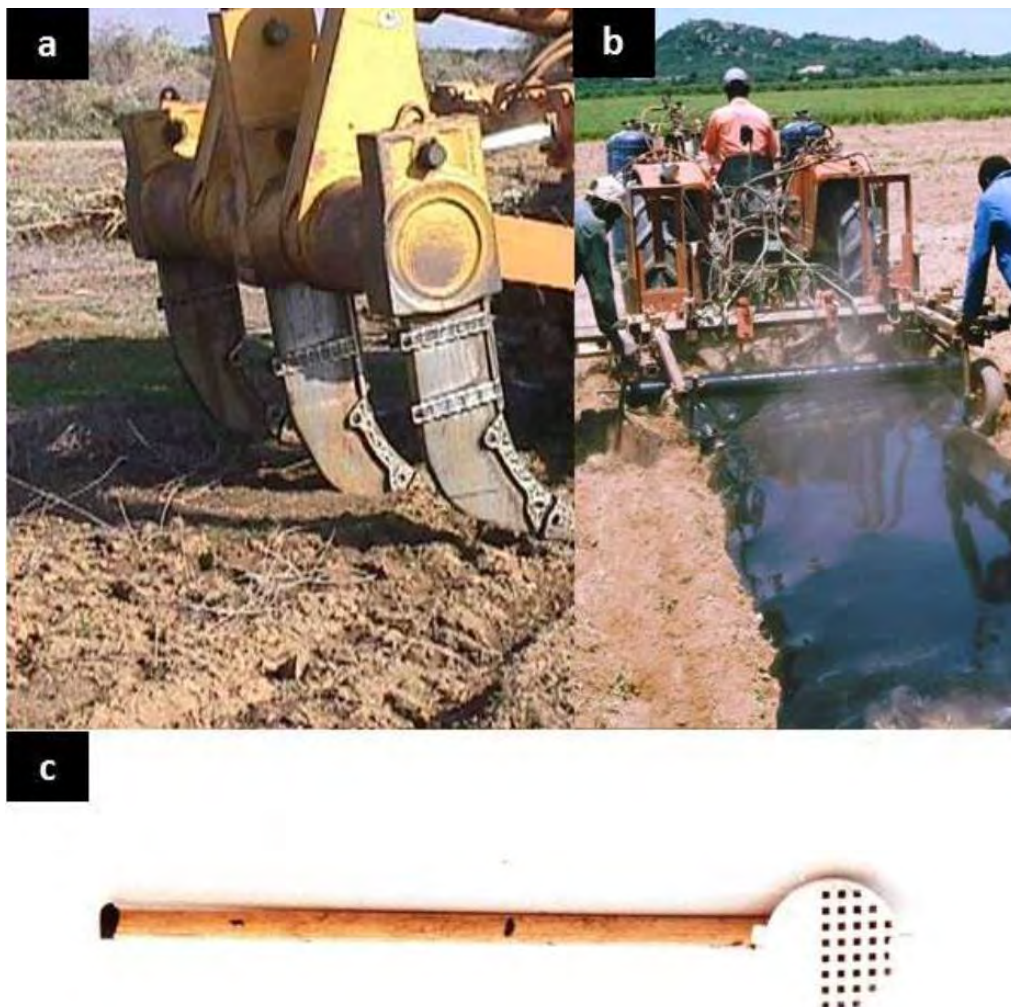
Figure 11.1. Soil preparation for methyl bromide soil fumigation.
a, Deep ripping with a D-10 Caterpillar bulldozer.
b, Tractor assisted plastic tarpaulin placement.
c, Wooden stake inoculum capsule prior to being inserted in the soil.

Figure 11.2. Manual placement of a clear fumigation plastic tarpaulin prior to 'hot gas', methyl bromide fumigation.

a, 'Lay flat' tubing that conveyed the heated methyl bromide under the tarpaulin laid out on a prepared soil bed.

b, Clear polyethylene 50 um plastic sheeting was manually laid out over the soil.

c, The plastic tarpaulin edges were sealed with soil and the lay flat tubing was attached to the header fumigant delivery pipes.
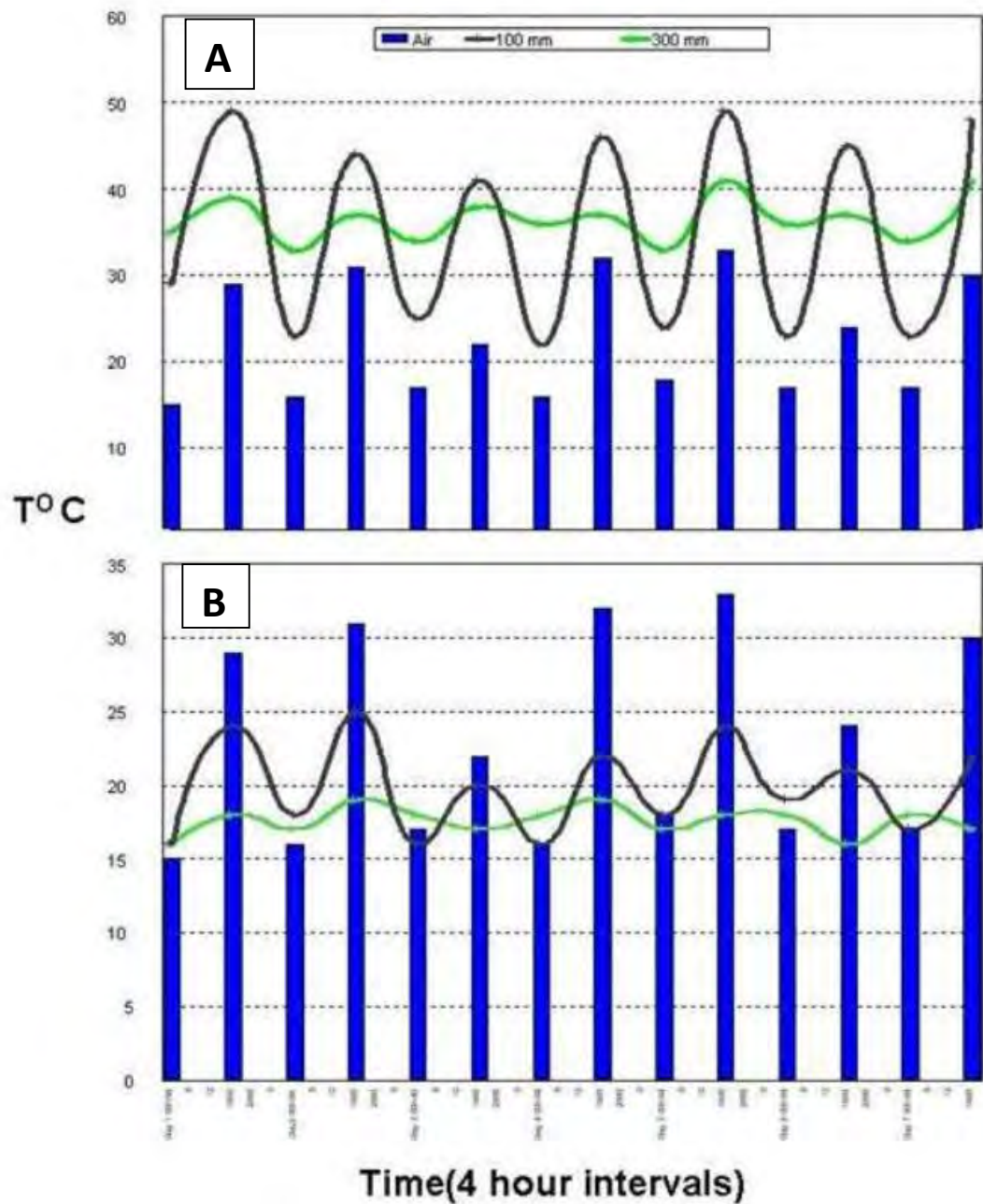
Figure 11.3. Air temperature, and soil temperatures at 100 mm and 300 mm depth. Both graphs represents the diurnal fluctuations in air and soil temperatures at the two assessed depths in the covered (tarpaulined) soil treatments (A) and the uncovered controls (B). Note the elevated soil temperatures dues to solarization effects.

Assessment of the inocula utilized in these fumigation studies indicated that it was viable at

the time of deployment in the fumigated plots. Soil fumigation treatments in all orchard sites were found to eradicate *N. psidii* inocula with the exception of the large root pieces (Table 11.2).

Table 11.2. Recovery of *N. psidii* from orchard soils naturally and artificially infested with *N. psidii*, following soil fumigation with methyl bromide of three guava orchards

Treatment	Mean percentage recovery of *N. psidii* [x]	
	Fumigated	Non-fumigated
Orchard 2		
Large Roots	57 b	100 a
Small Roots	0 a	82.5a
WS/M	0 a	100 a
Orchard 4		
Large Roots	87.5b	100 a
Small Roots	0 a	100 a
WS/M	0 a	100 a
Orchard 5		
Large Roots	100 b	100 b
Small Roots	0 a	92.5 b
WS/M	0 a	45.0 a

[x] 4 replicates per soil treatment, 10 replicate infected tissue samples per replicate.
Each figure represents the mean incidence of *N. psidii* across all the soil treatment replicates when assessed as the percentage of positive *N. psidii* recoveries per replicate.
Figures followed by the same letter in each column of each orchard, do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).
WS/M = autoclaved wheat seed plus mycelia of *N. psidii*.

N. psidii recovery was found to be efficient across all three types of inocula utilized at the three test sites, including the wooden stake inoculum capsules (Fig. 11.1). Methyl bromide soil fumigation significantly delayed the onset of GWD symptoms in two of the three orchard test sites as assessed at 36 months (Table 11.3).

Table 11.3. GWD incidence in trees planted into methyl bromide fumigated and non-fumigated soil at three orchard sites, with two guava cultivars

TREATMENT	**Months After Planting**									**Dead Trees[y]**	
	4	8	**12**	16	20	**24**	28	32	**36**	Guava cultivar	
			Incidence of GWD (%) [x]							FR	O
Orchard 2[z]											
Fumigated	0	0	0 a	0	0	0 a	40	40	40 a	**10**	**0**
Unfumigated	0	0	40b	80	80	100b	100	100	100b	**30**	**0**
Orchard 4											
Fumigated	0	0	0 a	0	0	0 a	60	60	60 a	**16**	
Unfumigated	0	0	60b	100	100	100b	100	100	100b	**30**	
Orchard 5[z]											
Fumigated	0	0	40 a	60	60	60 a	100	100	100a	**30**	**0**
Unfumigated	0	0	100b	100	100	100b	100	100	100a	**30**	**0**

[x] Incidence of GWD in the Fan Retief variety as measured by the appearance of symptoms in any replicate of a treatment, and scored as positive.

[y] This figure represents the total number of dead trees of each variety across all replicates at 36 months from the onset of the experiments.

[z] The trees utilized in the treatments conducted in Orchards 2 and 5 comprised of two varieties planted alternately in each replicate, Fan Retief and OT-1.

Figures followed by the same letter in each column of each orchard, do not differ from one another according to the Waller-Duncan k-ratio t test (P=0.05). Statistical analyses were conducted at 12, 24 and 36 months (**bold**).

FR = Fan Retief. O = Off Type 1 (OT-1). 5 replicates per soil treatment.

In all non-fumigated replicates, all Fan Retief trees were dead at 36 months (Table 11.3) at all sites, as compared to zero dead trees in the OT-1 trees (Orchards 2 and 5), across all treatments (Figure 11.4).
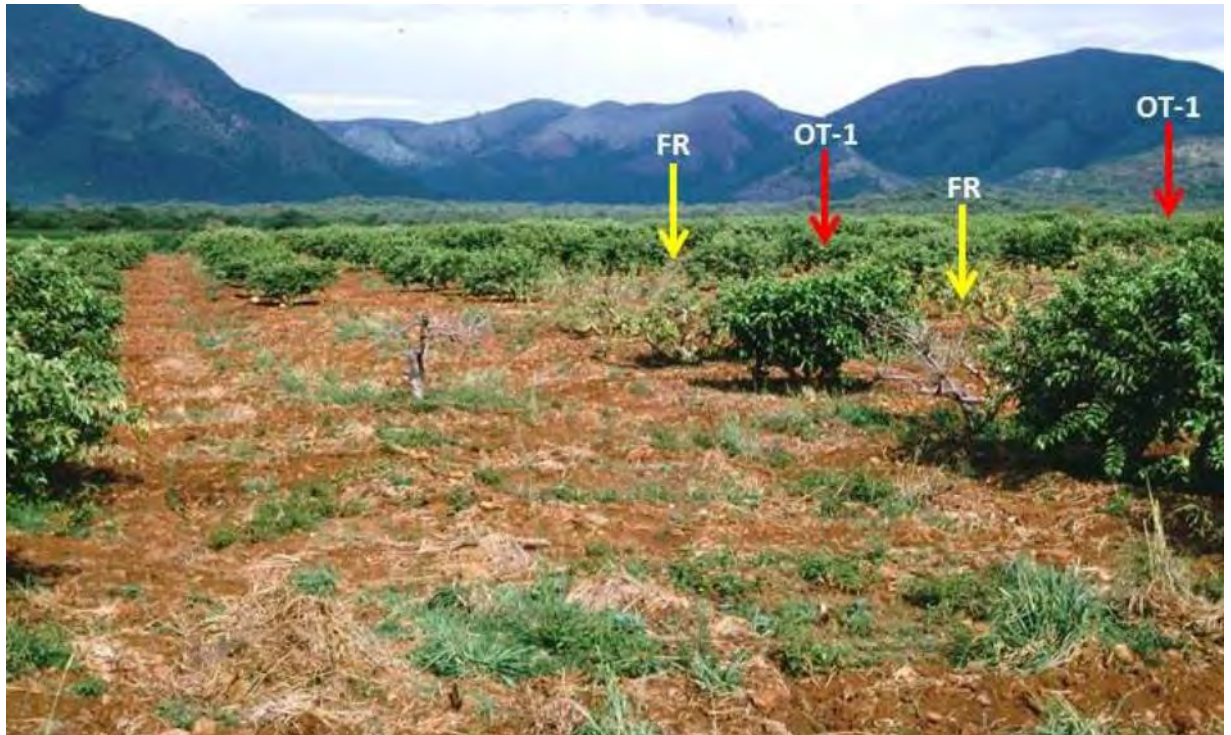
Figure 11.4. Orchard 2 fumigation, soil ripping and variety effects on the incidence of GWD. Note the wide tree row and column spacing due to annual soil ripping. Also note the diseased and dying Fan Retief guava trees (FR, yellow arrows) compared to the healthy resistant Off Type-1 trees (OT-1, red arrows).

When protective pesticides (fungicides and insecticides) were combined, with fumigation treatments, there was a significant reduction in plant mortality compared with all the non-fumigated treatment combinations (Table 11.4).

Table 11.4. The effect of foliar pesticide treatments on GWD incidence in trees planted into methyl bromide fumigated and unfumigated soil

Treatment	Incidence of GWD [x]									Total dead trees[y]
					Months					
	4	8	12	16	20	24	28	32	36	
Orchard 4										
F/P	0	0	0 a	0 a	0 a	0 a	20 a	20 a	40 a	**12**
Un/P	0	0	60b	100b	100b	100b	100b	100b	100b	**30**
Untreated	0	20	60b	100b	100b	100b	100b	100b	100b	**30**

[x] Incidence of GWD as measured by the appearance of symptoms in any replicate of a treatment and scored as positive.
[y] This figure represents the total number of dead Fan Retief trees across all replicates at 36 months from the onset of the experiments.
Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan k-ratio t test (P=0.05).
6 trees per replicate, 5 replicates per treatment. F/P = fumigated plus pesticide treatment. Un/P = Unfumigated plus pesticide treatment.

Disease expression in the fumigation/pesticide combination treatment was first observed at 28 months from the onset of these experiments. Nematode levels were reduced by the fumigation treatments at all sites and remained at low levels for the duration of the trial (Table 11.5).

Table 11.5. Incidence of the spiral nematode *Helicotylenchus dihystera* (Cobb) Sher in

rhizosphere soil of trees planted into methyl bromide fumigated and unfumigated soil at three orchard sites

| Treatment | Spiral nematode soil populations [x] | | | | |
| | Time interval before and after fumigation (months) [y] | | | | |
	-12	0[z]	12	24	36
Orchard 2					
Fumigated	6700a	8200 b	<500a	<500a	<500a
Unfumigated	5500a	6400 a	7200b	1100a	<500a
Orchard 4					
Fumigated	8900a	9300 a	<500a	<500a	<500 a
Unfumigated	9600a	10400a	6700b	5100b	2300b
Orchard 5					
Fumigated	4500b	3800 a	<500a	<500a	<500 a
Unfumigated	2900a	4800 a	3300b	4200b	<500b

[x] Soil nematode counts were rounded up to the nearest 100 and represent a mean value across all five treatment replicates.
[y] Soil nematode populations were assessed one year prior to treatment and annually thereafter for four years.
[z] '0' time was the point just prior (+/- 7 days) to fumigation.
Figures followed by the same letter in each column of each orchard, do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).

11.5   DISCUSSION

As with many other soil dwelling fungal plant pathogens, *N. psidii* was found to be highly sensitive to methyl bromide *in vitro*. An equivalent dose of 50 g.m^{-3} eradicated all propagules of *N. psidii* after a two day exposure (Table 11.1). This is consistent with other soil inhabiting fungal plant pathogens (Cebolla *et al.,* 1993). This laboratory assay provided a baseline dosage for field treatments of methyl bromide required to eradicate this pathogen from infected commercial orchards.

Use of plastic tarpaulins on the fumigation plots induced a significant solarization response in the soil, resulting in temperature maxima that were on average 26°C°C and 22°C°C higher (at the 100 mm and 300 mm soil depths, respectively) than in the non-tarpaulined plots. Sustained temperatures of between 41°C°C and 49°C°C were recorded at the 100 mm depth over the course of the fumigation period. The lethal upper temperature range for *N. psidii* is 44-46°C°C for an exposure period of 2 hours (Chapter 4). These soil treatments were deployed in the summer months, ensuring maximal infra-red radiation exposure and hence maximal thermal loading (Stapleton, 2000). Higher soil temperatures increase fumigant efficacy (Zhang *et al.,* 1998). However the tarpaulins also reduced the effects of rain in the treated soil profile, where water logging can reduce the efficacy of both soil solarization and methyl bromide fumigation (Zhang *et al.*, 1998; Shlevin *et al.,* 2004).

The tarpaulined soil fumigation treatments were effective at destroying the small roots and mycelia/wheat seed artificial inocula placed in the fumigation plots prior to the plots being fumigated. However, the large root inoculum was not destroyed by the fumigation treatments and this outcome emphasizes how critical it is to remove all large root pieces from the soil prior to fumigation.

In all three orchards where soil fumigation was tested, the development of GWD was retarded but by the end of the three year evaluation period, significant tree mortality had started to occur (Table 11.3) in these plots. When the fumigation treatments were combined with foliar fungicidal sprays there was a reduced rate of disease progression as compared to the fumigations treatments only (Table 11.4).

Soil fumigation with methyl bromide as a tool to manage GWD has serious limitations in terms of the rapid (2-3 years) re-emergence of GWD in the orchard; its risks, its costs and the global phase out of methyl bromide due to the compound's destructive effect on stratospheric ozone (Ohr *et al.,* 1996), impose significant constraints to its utilization.

In Orchards 2 and 5, where two varieties of guava were planted after fumigation, the tolerant/resistant OT-1 selection did not succumb to GWD whereas all the susceptible Fan Retief trees died (Table 11.3 and Fig. 11.4). This is an indication that the breeding for resistant cultivars is perhaps the most viable, and most cost-effective, solution to GWD.

As reported earlier in this study (Chapter 10), the spiral nematode is ubiquitous in the soils of guava production orchards in Southern Africa. Methyl bromide soil fumigation provided substantial and long lasting control of spiral nematode populations (Table 11.5). Previously the impact of the spiral nematode on growth and fruit yield in guava production was reported (Willers and Grech, 1986). These studies confirmed earlier experiments examining the potential of the spiral nematode to enhance the spread of *N. psidii* in guava orchards (Chapter 10).

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CHAPTER 12: EVALUATION OF CHEMOTHERAPEUTIC AND BIOLOGICAL AGENTS ALONE AND IN COMBINATION WITH METHYL BROMIDE SOIL FUMIGATION FOR EFFICACY IN GUAVA WILT DISEASE MANAGEMENT

ABSTRACT

Chemicals and known fungicides were screened *in vitro* for activity against *Nalanthamala psidii* (Biourge) Schroers and several exhibited robust inhibition against the pathogen. Field deployment of the selected chemotherapeutic agents resulted in significant reductions in guava wilt disease (GWD) progression in commercial orchards. The propiconazole injection treatment was the most effective single treatment. Potassium phosphite injection, benomyl injection, foliar thiophanate methyl and carbendazim foliar and soil drench treatments also reduced GWD progression and eventual tree mortality but at a lower level. Treatments comprising propiconazole injection alone or in combination with other treatments resulted in the lowest tree mortality over the course of 36 months. Basipetal movement and long residual activity (4-8 weeks) were characteristic of the propiconazole treatment. A selected biocontrol organism, *B. subtilis* Strain LC45, was found to have excellent *in vitro* activity against *N. psidii*, but provided little control when injected into field trees. Trunk injection was found to give rise to high levels of entry wound infection with *Polyporus occidentalis* Klotz [syn. *Coriolopsis occidentalis* (Klotzsch) Murrill], a white rot fungus. Extensive damage of the sap and heart wood was recorded in many injection treatments, the most severe being the potassium salicylate and *B. subtilis* treatments. Several effective biocidal wound dressings were assessed for post injection wound treatment, tentatively identifying some promising candidates. Curative or prophylactic chemotherapy with or without concurrent treatment with Methyl bromide, was not effective in managing GWD. Prophylactic use of fungicides coupled with improved durable resistance in commercial guava cultivars may however be prudent, particularly in the protection of wounds such as those that arise routinely during pruning.

12.1   INTRODUCTION

Nalanthamala psidii (Biourge) Schroers is a wound pathogen (Leu and Kao, 1979; Grech, 1985), infecting efficiently via pruning wounds (Chapters 9 and 10). Rhizosphere infection was also found to be significant in orchards, especially via root grafts (Chapter 11). The aim of this study was to evaluate fungicides for their potential to control or suppress guava wilt disease (GWD) under controlled and field conditions, testing stand-alone treatments as well as integrated treatments with Methyl bromide (MeBr) fumigation. A preliminary evaluation of biological control was also undertaken.

12.2   MATERIALS AND METHODS

12.2.1 Fungicide treatments

A large number of chemicals and fungicides (Tables 12.1 and 12.2) were screened *in vitro* for activity against *N psidii*. Solutions or suspensions of the fungicides were made in concentrations of 25, 50 and 100 mg.L^{-1} a.i., in a solvent mixture of ethanol and water at 1:1 v:v. Sterilized filter paper discs were placed in the fungicide solutions/suspensions, removed, allowed to dry and then two discs were placed on the surface of a plate of acidified corn meal agar amended with 250 mg.L^{-1} (CMA$_{aa}$), followed by a central inoculation of the media with *N. psidii*. Five replicates were used per treatment.

Cultures were incubated at 30°C°C in darkness and the growth of the fungus recorded over several days. In these studies only the most promising fungicide candidates were studied further, based on the efficacy of the chemical to suppress the growth of *N. psidii in vitro*.

12.2.2 Field fungicide applications

Field applications of chemotherapeutic agents were conducted in Orchards 2 and 5 (Chapter 5, Table 5.1). Established, healthy trees in a GWD infected orchard were selected. These trees were all at least 50-100m (10-20 trees) distant from any tree exhibiting symptoms of GWD. The treatments were as follows:

1. Bavistan® 50 WP [(carbendazim, 500g.kg^{-1}), BASF Pty Ltd, Johannesburg], applied around the base of the tree, covering an area of 4m^2, at a rate of 5g.m^{-2}, applied in 10 liters of water by watering can. In addition, this product was also sprayed on the foliage at a dilution of 100g in100 L water;

2. Bavistan® plus Temik® [(aldicarb, 50g.kg^{-1}), granular nematicide, Bayer Pty Ltd., Isando, RSA (formally Rhone Poulenc Pty Ltd., Johannesburg)]. Bavistan was applied as Treatment 1, whereas Temik was broadcast by hand at a rate of 20g m^2 to the base of the tree, covering an area of 4 m^2;

3. Lignisan® 25 EC [(benomyl, 250g.L^{-1}), injectable fungicide, Du Pont Pty Ltd, Johannesburg]. The product was diluted to a 10% v/v emulsion and applied via syringe injectors through four, 4mm x 50 mm holes drilled into tree trunk, approximately 0.5 m from the soil (Figs. 12.2 and 12.3), at a rate of 100 ml per tree by four syringes (25ml per each syringe), inserted around the trunk circumference, equidistant from one another. After injection, the holes were sealed with a bituminous graft sealing compound (Tree Seal ®, Protea Chemicals Pty. Ltd, Bryanston, South Africa);

4. Arbotech® 20 EC [(sodium hypophosphite 100g.kg^{-1}) and (2-(4-thiazolyl) benzimidazole 100g.kg^{-1}), Bayer Pty Ltd., Isando (formally Rhone Poulenc Pty Ltd., Johannesburg)]. This product was diluted to 10% v/v with water and applied by injection, as with Treatment 3;

5. Tilt® 250 EC [propiconazole (250g.L^{-1}), Syngenta Pty Ltd, Midrand, South Africa (formally

Ciba-Geigy Pty Ltd, Johannesburg)]. The fungicide was diluted to a 3 % (v/v) emulsion and injected into the trunks of the guava trees as with Treatment 3;

6. Tilt® 250 EC [propiconazole, Syngenta Pty Ltd, Midrand, South Africa (formally Ciba-Geigy Pty Ltd, Johannesburg)]. This was applied at 100 g in 100L water as a foliar spray.

7. Aliette® 70 WP [(fosetyl-Al 700g.kg^{-1}), Bayer Pty Ltd., Isando], applied as a foliar spray at 500g in 100 L water;

8. Potassium phosphite solution [(100g.L^{-1}), Merck Chemical, Halfway House, Johannesburg], applied as per Treatment 3, at a dose of 100 ml per tree;

9. Potassium phosphite solution (Merck Chemical Pty Ltd., Halfway House, Johannesburg) applied as foliar spray at a dose of 400g in100 L water;

10. Bayfidan® 25 E.C. [(triadimenol, 250g.L^{-1}), Bayer Pty Ltd., Isando], applied at a dose of 100g in 100 L water, as a foliar spray;

11. Topsin® 50% WP [(thiophanate methyl, 500g.kg^{-1}), Nippon Soda Co. Ltd, Tokyo, Japan], applied at 500 g in 100 L water as a foliar spray;

12. Potassium salicylate (Merck Chemicals, Halfway House, Johannesburg), injected as a 10 % w/w solution, as per Treatment 3;

13. Potassium salicylate applied as a foliar spray at a dose of 500g in 100 L water;

14. *Bacillus subtilis* Strain LC 45 was injected into trees. The cell count was adjusted to 3.5 x 10^8 c.f.u's per ml of water. 100 ml of this suspension was injected into each tree, utilizing the same methodology as in Treatment 3.

15. The treatment combined Treatments 1, 5 and 8, i.e., a drench and foliar treatment with carbendazim plus injection applications of propiconazole and potassium phosphite. The two injection treatments were applied via separate holes in the trunk**;**

16. Untreated Control.

Foliar treatments were applied immediately after annual pruning and repeated every 4 months. Injection treatments were applied two weeks before pruning and repeated every 6 months (mid-spring and mid-autumn).

All the spray treatments were diluted in 100 liters of water and the pH adjusted to 6.5 with citric acid, prior to applying a full cover spray (equivalent of approximately 3,000 L.ha^{-1} spray volume or approximately 4 L per tree) to experimental trees by a motorized vehicle mounted hand sprayer operating at 150 psi. All sprays contained 0.01 % (v/v) of the spreader/sticker, Latron B$^®$ (Dow Agrosciences, Indianapolis, USA). Care was taken to ensure that the whole tree and inner branch canopy was thoroughly covered and that the soil below the canopy was also exposed to these applications.

To determine the probable distribution patterns of the injected liquid in the woody tissues of guava trees, a blue dye was injected into the trunk of a selected guava tree by drilling into the trunk, a 5 mm diameter hole positioned approximately perpendicularly to the vertical plane, to a depth of 50 mm and inclining approximately 20-30° upwards from the horizontal plane, using a standard drill bit and a 12V operated hand drill, (Fig. 12.2a). Holes were drilled not less than 300 mm above the soil line, and each hole was positioned (as far as possible) in line with the Northern cardinal point. 25ml of sterile distilled water (STW) with 10ml.L^{-1} of a standard blue food colorant added, was drawn into a 60 ml capacity syringe, followed by 30 ml of air (Fig. 12.3). The syringe was fitted with a modified tip, tapering to an outer diameter of 3 mm, and

this was inserted into the drilled hole in the tree trunk. The contents of the syringe were pressurized to three atmospheres by a series of evenly spaced locking holes in the casing and plunger. When the desired pressurization was reached, a nail was inserted into these aligned locking holes thereby establishing the desired initial pressure in the syringe. 30 minutes after the syringe was completely discharged, the trunk was sectioned in 10 cm increments above and below the injection holes until there was no visual detection of the dye. The procedure was refined prior to field deployment by utilizing recently cut main trunks and scaffold branches and applying the injection solution as described above (Fig. 12.2c).

The incidence of GWD was monitored over 3 years in all experiments at the two locations. Trees were pruned once per year and the pruning wounds immediately treated with a mixture of Cupravit® 80WP [(copper oxychloride, 800g.kg^{-1}), Bayer Pty Ltd., Isando, RSA] at 5g.L^{-1} water, and Captan® 80 WP [(N-trichlorormethylthio-4-cyclohexane-1,2-dicarboximide, 800g.kg^{-1}), Syngenta Pty Ltd, Johannesburg] at 1g.L^{-1} water. The sprays contained 0.01 % (v/v) of the spreader/sticker, Latron B®. The materials were applied by a tractor mounted hand sprayer at a rate of 2200 L.ha^{-1}. All treatments received these pruning wound protectant sprays. Twelve single tree replicates were used per treatment. Two trees in each treatment were used for destructive tissue sampling comprising of collecting root and shoot tissue samples from these trees at 1 week, 4 weeks, and 8 weeks after treatment. After the last samples were collected from these trees, they were no longer used in these trials.

The distribution of the chemotherapeutic agents in the sampled trees following treatment was assessed by extracting 4mm diameter by 50mm in length, wood cores from the main trunk,

355

primary roots and scaffold branches. After sampling, the wounds were treated as above by spraying them with the same fungicide spray as used to protect the pruning wounds (as described earlier) and sealing the holes with Tree Seal $^®$. The collected samples were immediately refrigerated and processed within 24 hours, by surface sterilizing the entire core with 20 ppm hypochlorous acid solution for 20 seconds, gentle rinsing so as to not fragment the sample and placing it on CMA$_{aa}$ aligned on the diameter of the Petri plate. Immediately following the core placement on the Petri plate, a 3 mm deep by 40 mm long channel was excavated in the agar medium parallel to the guava tissue core, at a distance of approximately 20 mm. A spore suspension (7 x 10^6 conidia/ml) of *N. psidii* was introduced into this channel, filling it to approximately 50 % of the volume. Cultures were incubated at 30°C°C in darkness for 10 days and then assessed for microbial growth towards the guava tissue core.

12.2.3 Biological Treatments

Bacillus subtilis LC-45 was isolated from main trunk xylem vessels of a healthy guava escape tree (in a diseased section of Orchard 2), and was cultured in autoclaved peptone complete nutrient broth (Difco, Sparks, Nevada) plus 0.5 yeast extract (Marmite$^®$, Unilever Pty Ltd, U.K.) and 0.1 % dextrose. The media pH was adjusted to 7.2 with 1M NaOH. The bacterium was cultured in 500 ml conical flasks for ten days at 30°C°C. Cultures were agitated by continuous rotary shaking. Cells were harvested by centrifugation at 10,000 g for 5 minutes. Most of the supernatant was removed and the cells were re-suspended in a solution of 3% polyethylene glycol in sterile distilled water and the cell concentration adjusted to a final density of approximately 3.5 x 10^8 c.f.u. ml^{-1}. This material was used to inject trees as described

earlier. Each mature tree received 4 x 25 ml trunk injections of the cell suspension every six months for three growing seasons. Trees were pruned once per year and the pruning wounds were immediately treated in the same manner as for the chemical treatments described above.

In both the chemotherapeutic and biological injection treatments, at the termination of the experiments (or tree death induced by GWD), five trees from each of the injection treatments were selected and initially sectioned in the plane of the injection hole. Subsequent sections were in the transverse (horizontal) plane in 10 cm increments acropetally and basipetally from the injection points in the trunk and extending in both directions for 50 cm. Finally, the trunk sections were cut longitudinally along the vertical center plane to determine the acropetal and basipetal wood necrosis and possible infection by wood rotting microbes. Wood sections were surface sterilized with a 20 ppm hypochlorous acid solution, rinsed, placed in sealed plastic bags and incubated in darkness at 30°C°C. Sections were observed daily for the presence of fungal growth. If detected, sub cultures were made onto PDA and identified. If a microbe was isolated with a high frequency (>50%) from wood sections, then fulfillment of Koch's postulates were attempted on 10 year old clonal trees of cv. Fan Retief, by trunk inoculation using drilled holes as described above, as the inoculation point.

Chemical weed control was used exclusively in all the experimental blocks. In Orchard 2, chlorination (5 ppm free chlorine) of the irrigation system serving the treatment areas was implemented by installing an in-line injection pump and injecting sodium hypochlorite into the main irrigation line immediately after being pumped out of the holding reservoir. Chlorination of the irrigation system was not conducted at Orchard 5, as the water source emanated from a

130 m deep borehole well and was not considered to be a likely source of infection.

12.4   DATA ANALYSES

Data were analyzed by one and two way analysis of variance and where appropriate, means were separated using the Waller-Duncan k-ratio t test (Little and Hills 1990).

12.5   RESULTS

12.5.1 Fungicide treatments

In vitro screening of numerous fungicides for efficacy against *N. psidii* indicated that several of the agrochemical groups inhibited mycelia growth of *N. psidii* (Fig. 12.1 and Table 12.1). Neither potassium salicylate nor the phosphorous acid based fungicides (Aliette[®] and potassium phosphite) exhibited activity *in vitro* against *N. psidii*. The isolate *Bacillus subtilis* Strain LC 45 substantially inhibited the growth of *N. psidii in vitro*, as well as inducing the production of a red pigment. (Fig. 12.1).
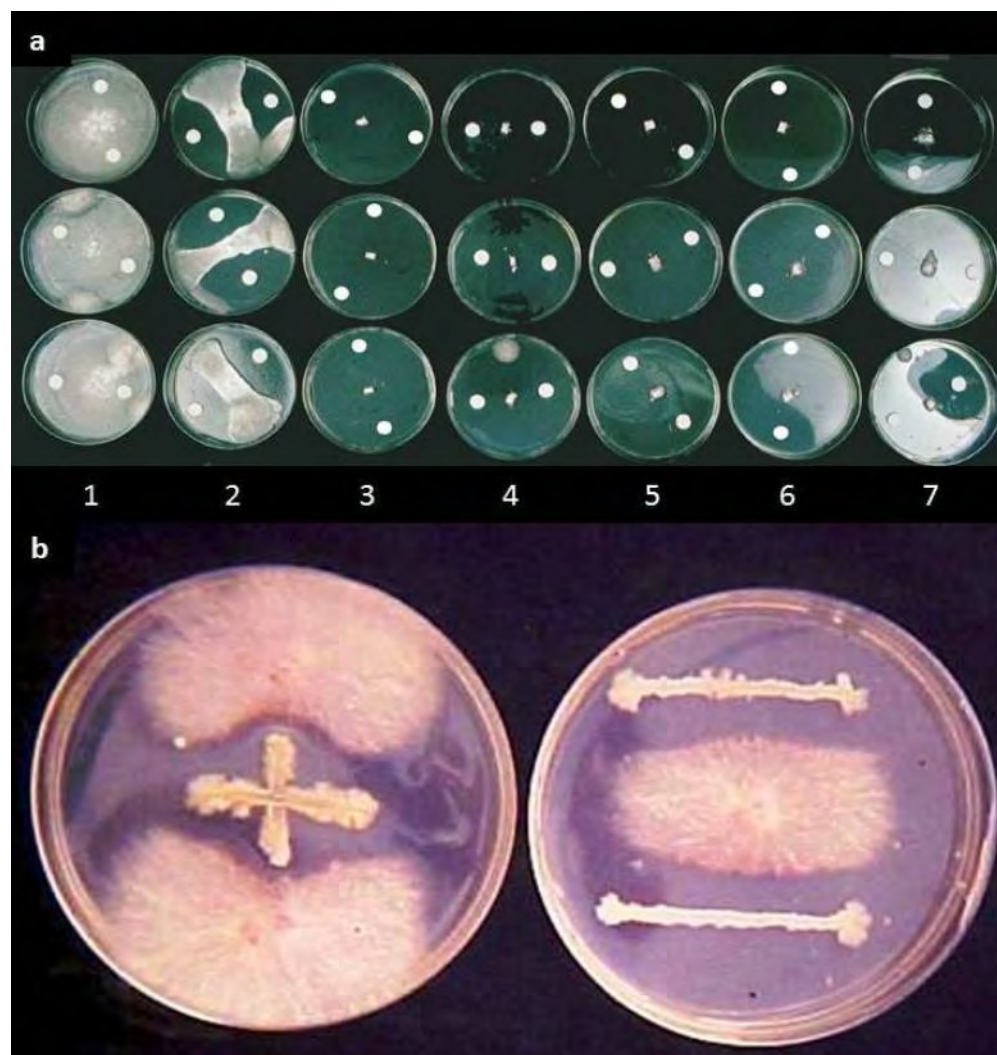
Figure 12.1. *In vitro* assessment of chemical and biological agents against *Nalanthamala psidii*.
a. Agar plate screening of various fungicides for inhibition of *N. psidii* mycelial growth.
1 = control; 2 = Bravo; 3 = Bavistan; 4 = Lignisan; 5 = Propiconazole; 6 = Bayfidan; 7 = Topsin.
b. Bacterial antagonism by *Bacillus subtilis* Strain LC 45 against *N. psidii*. Note the red pigmentation in the advancing front of *N. psidii* mycelia.

Table 12.1. *In vitro* screening of fungicides and selected compounds for suppressive activity

against the guava wilt pathogen, *Nalanthamala psidii*

Treatment[w]	Fungicide or chemical active ingredient concentration (mg.L^{-1}) [x]		
	25	50	100
	Percentage inhibition of mycelial growth [y]		
Bavistan	73 cd	86 bc	100 c
Lignisan	100 e	100 c	100 c
Arbotech	59 c	100 c	100 c
Propiconazole	100 e	100 c	100 c
Aliette	0 a	0 a	23 b
Bayfidan	100 e	100 c	100 c
Bravo	24 b	57 b	100 c
Topsin	85 de	100 c	100 c
Potassium Phosphite	0 a	0 a	15 ab
Potassium Salicylate	0 a	0 a	0 a
Control	0 a	0 a	0 a

[w] Suppliers for the compounds listed are described earlier in this Chapter.
[x] All compounds were diluted to the specific values in terms of their active ingredient concentration.
 Petri plate *in vitro* inhibition of mycelial growth was assessed by determining the total area of fungal growth in each treatment as compared to the controls and expressing this as a percentage. This value was averaged over the five treatment replicates.
 Figures followed by the same letter in each column, do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).

12.5.2 Field fungicide applications

Large differences in field efficacy in control of GWD were observed as a result of the 15 treatments (Table 12.2). Treatments 5 and 15 resulted in the lowest tree mortality and the least disease in the treated plots across all treatments at 36 months from the onset of the experiment.

Treatments 1, 2, 3, 8 and 11 also had some effect, causing significantly lower disease levels than the Untreated Control, but still at higher levels than those resulting from Treatments 5 and 15. The biocontrol treatment tested was not effective in the field in suppressing GWD.

Table 12.2. Field evaluation of selected biological and chemical compounds for suppressive activity against the guava wilting pathogen, *N. psidii,* in two commercial orchards over a three year evaluation period

Treatments	Incidence of GWD [x] Time (Months)								Tree death[y]
	8	12	16	20	24	28	32	36	
1, Bavistan SD & F.	0	0	0	20	20 *	30	45	45 *	**(9)**
2, Bavistan SD & F plus Temik.	0	0	0	5	15 *	30	30	40 *	**(8)**
3, Lignisan inj.	0	0	0	0	0 *	20	20	45 *	**(9)**
4, Arbotech inj.	0	20	45	60	70	70	70	70	**(14)**
5, Propiconazole inj.	0	0	0	0	0 *	0	25	25 *	**(5)**
6, Propiconazole F.	0	0	0	35	35 *	50	55	70	**(14)**
7, Aliette F.	0	25	45	60	85	100	100	100	**(20)**
8, P. Phosphite inj.	0	0	0	0	25 *	25	40	40 *	**(8)**
9, P. Phosphite F.	0	0	0	0	10 *	40	65	65	**(13)**
10, Bayfidan F.	0	0	0	0	0 *	35	50	65	**(13)**
11, Topsin F.	0	0	0	10	40	40	55	60 *	**(12)**
12, Potassium Salicylate inj.	0	35	35	45	60	60	80	95	**(19)**
13, Potassium Salicylate F	0	0	45	60	60	100	100	100	**(20)**
14, *B. subtilis inj.*	0	0	25	25	35 *	50	75	100	**(20)**
15, 1 + 5 + 8	0	0	0	0	0 *	0	0	25 *	**(5)**
16, Untreated	15	25	40	55	70	100	100	100	**(20)**

[x] As assessed by the appearance of any known symptom of GWD. Figures above are averaged across the two experimental sites (Orchards 2 and 5). Ten single tree replicates assessed per treatment.

[y] Total number of dead trees in each treatment (sum of the two experiments).

* Indicates values in the same column as being significantly different from the Untreated (16), according to the Waller-Duncan k-ratio *t* test (P=0.05).

N.B. GWD was absent in all treatments at the 4 month assessment period and hence these data were not included in this table. SD = soil drench; F= foliar spray; inj = trunk injection.

Initially, (Week 1, Table 12.3), many of the treatments produced inhibitory concentrations of compounds in the excised tissues.

Table 12.3. Bioassay detection of qualitative mycelia inhibition factors in excised tissues from treated guava trees

Treatments[w]				Time [x]					
	Week 1			**Week 4**			**Week 8**		
	MB	T	MR	MB	T	MR	MB	T	MR
1, Bavistan SD & F.	+	-	-	+	-	-	-	-	-
2, Bavistan SD & F. plus Temik.	+	-	-	-	-	-	-	-	-
3, Lignisan inj.	+	+	-	+	+	-	+	+	-
4, Arbotech inj.	+	-	+	-	-	-	-	-	-
5, Propiconazole inj	+	+	+	+	+	+	+	+	-
6, Propiconazole F.	-	-	-	-	-	-	-	-	-
7, Aliette F.	-	-	-	-	-	-	-	-	-
8, P. Phosphite inj.	-	-	-	-	-	-	-	-	-
9, P. Phosphite F.	-	-	-	-	-	-	-	-	-
10, Bayfidan F.	+	+	-	-	-	-	-	-	-
11, Topsin F.	+	+	-	-	-	-	-	-	-
12, Potassium Salicylate inj.	-	-	-	-	-	-	-	-	-
13, Potassium Salicylate F.	-	-	-	-	-	-	-	-	-
14, *B. subtilis inj.*	+	+	-	-	-	-	-	-	-
15, 1 + 5 + 8	+	+	+	+	-	-	+	+	-
16, Untreated	-	-	-	-	-	-	-	-	-

[w]As described earlier in this Chapter.
[x] Tissue samples were taken from two surplus (boundary) trees per replicate at various weeks (as indicated above) after the commencement of the experiments. Three tissue samples per tree were plated and assessed for fungitoxic exudates at each interval.
'+' , indicates the presence of mycelia inhibition in the Petri plate cultures whereas '-' , indicates no inhibition, both as compared to the Untreated control.
Data are averaged across the two experimental sites (Orchards 2 and 5).
MB = main branch; T = main trunk; MR = main root.

Injected propiconazole was the only treatment that generated mycelial inhibition from the main root core samples, in the agar challenge tests, four weeks after treatment (Table 12.3). By week 8, inhibitory components were still detected emanating from trunk and main branch cores from Treatments 3 (Lignisan), 5 (Propiconazole injected) and 15 (a combinational treatment comprising Treatments 1, 5 and 15, which also utilized injected propiconazole). At week 8, no further fungitoxic components were detected emanating from main root core samples. Injection of propiconazole was found to distribute the compound more effectively, both temporally and spatially in guava tissues (Table 12.3), than when the fungicide was sprayed onto the foliage.

The injection of chemicals into the guava trunk was found to be rapid with solutions, but substantially slower with suspensions. The drilling and injection procedure was found to take a little over two minutes per tree (Figs. 12.2a and 12.3).

Figure 12.2. Chemical delivery to guava trees by trunk injection.
a. A 12 volt portable drill with a 4 mm drill bit excavating a 50 mm deep hole in the main trunk.
b. Distribution patterns of a blue dye when injected into the trunk of a guava tree, 30 cm above the injection point.
c. Distribution patterns of a blue dye injected into the excised trunk of a guava tree, 5 cm above the injection point.

Fig. 12. 3. Syringe preparation prior to tree injection (left). Note the volume of air in the syringe that allows for pressurization of the syringe chamber. Syringes positioned and being pressurized prior to locking (right). Note the upward inclined angle that allows the liquid to accumulate towards the tip of the syringe.

The distribution pattern of the injected chemicals was found to generate a globular pattern at and around the point of injection (Fig. 12.2), whereas 30 cm above or below the point of injection, a series of overlapping crescent shaped distribution patterns were evident (Fig. 12.2b). The blue dye could be traced in the sectioned tree for approximately 55 cm acropetally and 40 cm basipetally from the point of injection.

At the termination of the experiments, a large degree of wood damage was observed in tissues around the injection sites of the five assessed trees that were destructively sampled, from each treatment. The damage consisted of chemical damage to the immediate exposed tissue, extending three dimensionally into the heartwood of the tree, emanating from the injection hole as well as extensive vertical tissue damage (Figs. 12.4 and 12.5).
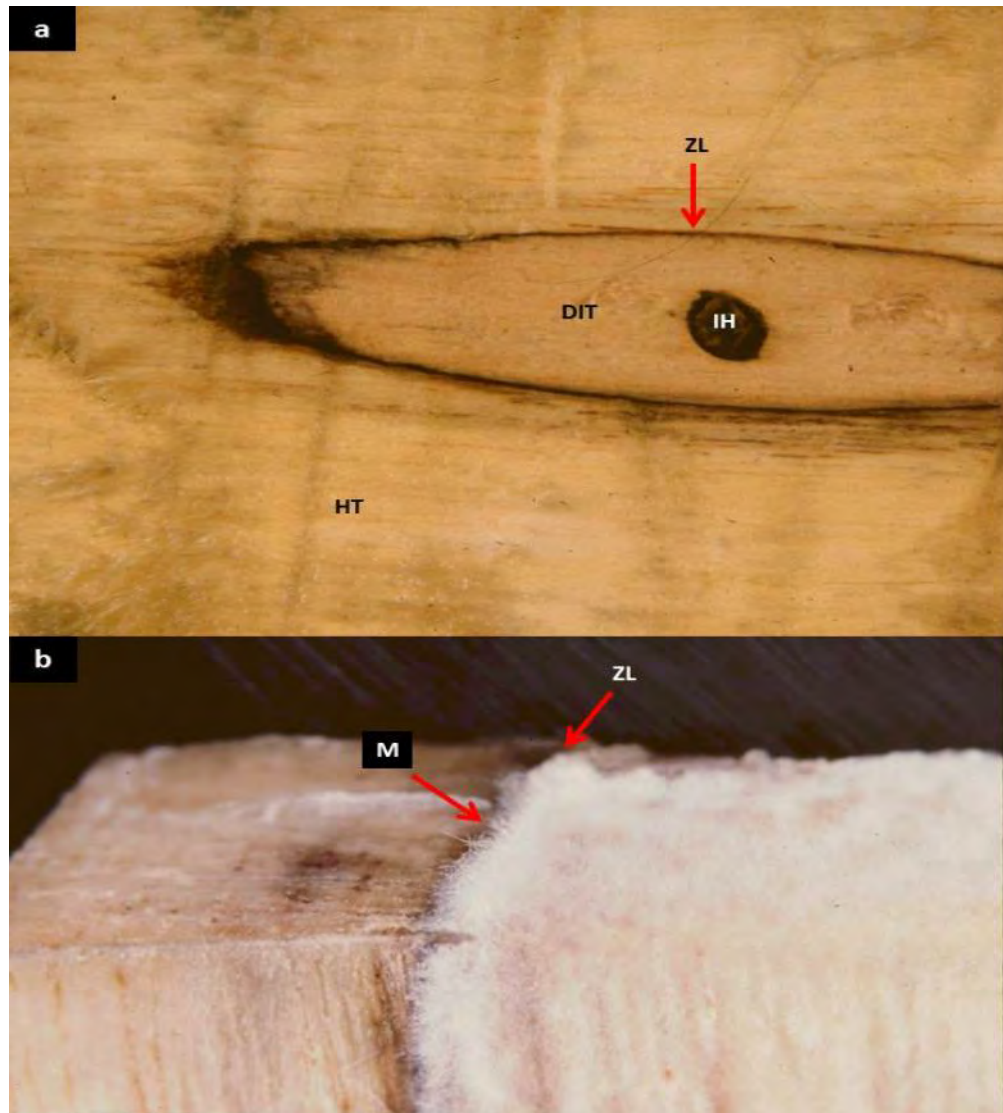
Figure 12.4. Main trunk injection damage to guava.
a. Longitudinal section of trunk xylem tissue from the injection site.
b. Transverse section of trunk xylem tissue from the injection site. The wood section was incubated for three days at high humidity. Actively growing mycelia (M) can be seen emanating from just behind the fungal zone line (ZL) in the dead infected tissue.
IH = injection hole; DIT = dead infected wood tissue; ZL = fungal zone line at the boundary of healthy and dead wood; HT = healthy tissue.
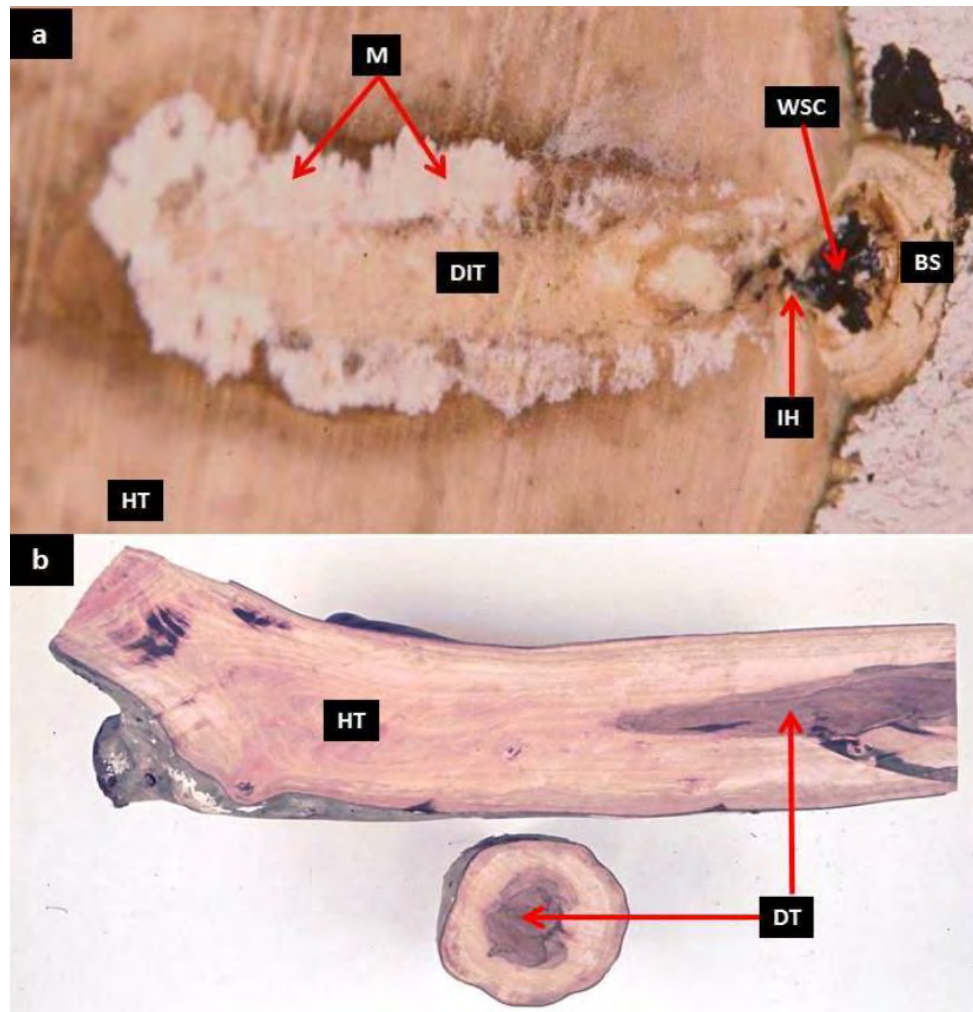
Figure 12.5. Injection damage in guava trunks.
a. Transverse section of the main trunk along the plane of the injection site.
Note the bark sealing the injection wound and the remnants of the wood sealing compound. The wood section was incubated for three days at high humidity and actively growing mycelia can be seen emanating from the boundary of the damaged tissues.
b. Longitudinal section above the injection point (approximately at the mid-point of the top longitudinal trunk section on the extreme right of the photograph). The dead tissue has extended into the heartwood. This trunk section as shown, received Treatment 4 (Arbotech® 20 EC).
IH = injection hole; DIT = dead infected wood tissue; DT = dead tissue; WSC = wood sealing compound; HT = healthy tissue; BS = bark growth over injection hole; M = fungal mycelia.

Microbial damage from the opportunistic fungal colonist *Polyporus occidentalis* Klotz [syn.

Coriolopsis occidentalis (Klotzsch) Murrill] was identified and enumerated (Figs.12.4 and

12.5; Table 12.4).

Table 12.4. Assessment of trunk wood damage in field guava trees treated with injected
therapeutic agents

| Treatments[w] | Wood damage [x] | | | *P. occidentalis* |
| | Horizontal (cm^2) | Vertical (cm) | | recovery[y] |
		(A)	(B)	
(3) Lignisan inj.	8.7	10.5	6.3	0
(4) Arbotech inj.	19.2	27.5	13.7	46
(5) Propiconazole inj.	6.9	8.4	10.6	12.5
(8) P. Phosphite inj.	9.3	6.7	5.1	17
(12) Potassium Salicylate inj.	14.7	22.4	13.2	83.3
(14) *B. subtilis inj.*	5.8	5.4	3.8	100
(15) Bavistan S&F/ Propiconazole inj/ phosphite inj [z]	11.7	12.9	8.8	0
(16) Control	0	0	0	0

[w]All treatments from Table 12.3 that were applied by injection of the tree trunk. Figures in
parentheses are treatment numbers from Table 12.3.
[x] Wood damage was assessed in terms of the mean area of the necrotic tissue in the horizontal
plane of the injection holes from the 5 assessed trees per treatment. Values represent the
calculated mean lesion area in the horizontal plane and the mean maximum linear necrotic
distribution in the vertical plane of the assessed trees.
[y] Mean incidence of *P. occidentalis* recovered from injection holes across all years in the
assessed trees, expressed as a percentage recovery from the total number of the injection holes.
[z] Total number of injection holes/tree over three seasons = 48 (this treatment consisted of two
injected compounds). A = acropetal extension; B = basipetal extension.

The pathogen was successfully isolated in pure culture and the fulfillment of Koch's postulates was confirmed by inoculation and recovery of the fungus from 10 year old guava trees. In some cases, the bark sealed over the injection hole and fungal colonization was still evident (Fig. 12.5a) in the excised sections, as observed in many of the field injection wounds. Wood damage as assessed in the five tree subset from each treatment and was found to be most severe from Treatments 4 (sodium hypophosphite and thiabendazole), 12 (potassium salicylate), 14 (*B. subtilis*) and 15 (carbendazim, propiconazole and potassium phosphite). Extensive tissue necrosis was discovered emanating from the injection wounds and was particularly evident in Treatments 4, 12 and 14 which were also found to be infected with *P. occidentalis* to various degrees (46, 83.3 and 100 % respectively). *P. occidentalis* was not recovered from damaged wood tissues in trees receiving Treatment 15.

In other injection treatments, lower levels of infection by *P. occidentalis* (12.5-17 %) of the injection holes were found (Table 12.4). In addition, injection holes in field trees that had received Treatments 4, 12 and 14 were observed to show evidence of fungal mycelium emanating from the injection holes by Year Three, whereas the other treatments did not show this high incidence (approximately < 5 % of the trees receiving the other injection treatments exhibited this phenomenon). Besides the direct toxicity of some of the chemotherapeutic agents to the guava wood tissue [Arbotech (Fig. 12.5)], it was observed that the injection wound infections by *P. occidentalis*, were usually associated with two predisposing factors: injection holes that had an acute downward incline (and hence could accumulate water) and injection holes that were regularly exposed to water (irrigation or rainfall).

12.5.3 Biological Treatments

Bacillus subtilis LC-45 did not have any positive effect on GWD. The onset of symptom expression was not delayed. Tree mortality was the same as the Untreated controls.

12.6   DISCUSSION

Nalanthamala psidii was found to be susceptible *in vitro* to a number of fungicide chemical classes including the triazole and benzimidazole groups (Fig. 12.1 and Table 12.1), as well as the biological agent, *B. subtilis* Strain LC45 (Fig. 12.1). Phosphite and salicylate based chemistries exhibited little of no *in vitro* effect on mycelial growth of the fungus, which was to be expected, as these materials have a mode of action primarily by induction of the host plant's own systemic acquired resistance (SAR) system (Kessman *et al.*, 1994). Therefore they would have minimal or no direct impact on this fungus at the rates utilized in these experiments. However these compounds were included in the field trials due to their ability to elicit inducible host defense mechanisms primarily through SAR metabolism (Benhamou and Bélanger, 1998; Daniel and Guest, 2006). Inducible host resistance is increasingly deployed as part of integrated disease management strategies across a broad range of pathogens (Vallad and Goodman, 2004). Recently (Araujo, *et al.*, 2015) reported activity of SAR inducing compounds against a serious vascular wilting disease of mango, caused by *Ceratocystis fimbriata* Ellis and Halst. Previously other studies have indicated activity of SAR inducing chemistry on other vascular wilting pathogens (Ribeiro, *et al.*, 2006) such as on Cacao wilt caused by *Verticillium dahliae* Kleb. In these studies, treatments comprising of potassium phosphite injections (Treatments 8 and 15) did suppress GWD symptom development and ultimately reduced the rate of tree death, but not at a commercially acceptable level. Future studies integrating SAR inducing compounds

together with varieties that have higher levels of genetic tolerance will be highly beneficial in elucidating commercial management strategies for GWD.

When applied in the field, some of the chemotherapeutic agents were found to significantly reduce the rate of disease progression in commercial orchards. The propiconazole injection treatment was the most effective singular treatment in terms of GWD suppression and retardation of the spread of the disease at the experimental sites (Table 12.2). Treatments that also reduced the development of GWD were the potassium phosphite injection (Treatment 8), the benomyl injection (Treatment 3), foliar thiophanate methyl (Treatment 11) and the carbendazim foliar and soil drench treatments (Treatments 1 and 2). The combination of three treatments (Treatment 15, Table 12.2), was relatively effective in retarding the onset of GWD symptom expression and ultimately resulting in the lowest tree mortality across all treatments. This treatment combination was generally more effective than the individual standalone treatments. Treatment 15 reduced tree death rate by over 75% compared to the untreated trees.

Inhibition of *N. psidii* by excised tissues from the fungicide treated guavas indicated that treatments containing propiconazole and injected into the trunk appeared to move both acropetally and partially basipetally (Table 12.3). Foliar applied propiconazole did not exhibit these attributes. Propiconazole (as well all azole chemistry fungicides) are described as moving acropetally in plants and being generally translaminar in terms of leaf tissue translocation (Mueller *et al.,* 2008). In these studies, trunk injected propiconazole was the most effective fungicide for GWD management. This may in part be due to the longer persistence in plant tissues as well as the product's downward movement in the tree following trunk injection

(Table 12.3). Propiconazole has been used effectively for the control of *Armillaria* root rot and this was also partly due to the downward movement of the fungicide in plant xylem tissues (Amiri *et al.,* 2012). The assessment of the distribution of the applied chemicals was made on the basis of the presence or absence of fungitoxic components in the excised wood cores. However it was observed that differences did exist in the degree of inhibition of *N. psidii* mycelia in the agar Petri plate assays. These differences are likely attributable to not only the ability of the chemicals to distribute in the woody tissues, but also the chemistry, degradability and diffusibility in xylem and agar.

These variables were beyond the scope of these studies, but requires further investigation in order for this bioassay technique to be useful in the elucidation and enumeration of the kinetics of distribution in guava tissues of these compounds.

Dye injection into the main trunk of the guava trees indicated a much greater acropetal movement than basipetal movement by water soluble components (Fig. 12.2). In these studies the dye moved approximately 40 cm downwards from the point of injection. This effect may be consistent for water soluble materials, but is unlikely to be similar for low water solubility or lipid soluble materials such as many of the triazole and benzimidazole fungicides. Propiconazole is unusual in that it has a higher water solubility (110 mg.L^{-1}) than most comparable fungicides such as benomyl (3 mg.L^{-1}) or epoxiconazole (7 mg.L^{-1}). This may account for the findings in this study and others where propiconazole exhibits basipetal movement in xylem tissues that are generally most uncharacteristic of azole chemistry fungicides (Appel and Kurdyla, 1992; Kenna, 1995; Mertoglu *et al.,* 2011).

Trunk injection is an efficient tool for the application of compounds directly into the xylem tissues of trees (Appel and Kurdyla, 1992), including guava, in agreement with these studies. Given that *N. psidii* is a wound pathogen that colonizes guava xylem tissues, direct delivery of chemotherapeutic agents into these tissues would seem a logical deployment method. However, in these studies, one negative side effect of these injections was the many opportunistic infections by the wood rotting basidiomycete, *Polyporus occidentalis* Klotz [syn. *Coriolopsis occidentalis* (Klotzsch) Murrill]. This fungus is a white rot fungus that is a competent wood rotting pathogen of a wide variety of trees (Gilbertson, 1980; Kovacs, 2001). The fungus was found to infect the injection holes, and to colonize chemically killed wood adjacent to the wound, from which it slowly colonized infected healthy xylem (Figs. 12.4 and 12.5). In most cases where injected chemicals had a high phytotoxic impact in the surrounding tissue around the injection hole, then fungal infection was severe. Surprisingly, *P. occidentalis* was found to infect (albeit to a lesser degree) these wounds even though there should have been an inhibitory residue of the various fungicides in and around the injection hole, especially with propiconazole which is active against Basidiomycetes. Injection holes that had a propensity to accumulate water were much more likely to become infected by *P. occidentalis*. This may have led to fungicide leaching and/or microbial or chemical (or both) decomposition of the fungicide. The water accumulation was generally the result of rainfall or irrigation (when the holes where within the irrigation distribution pattern, as was the case in Orchard 5, which had an overhead sprinkler irrigation system). Sealing the holes with a non-biocidal graft sealant (Tree Seal®) did not alleviate the malady, and in many cases mycelium of *P. occidentalis* was found in the hole under the sealant. It was also observed that in some cases the bark over grew

the injection hole even though it was infected (Fig. 12.5). This indicated that the infection by *P. occidentalis* probably occurred at or near the time of the injection, which could have been the result of inadvertently contaminating the injection equipment with soil or plant debris. *Polyporus occidentalis* is an opportunistic wound pathogen, colonizing dead wood that usually forms an infection platform into healthy tissue (Schmidt and Czeschlik, 2006). Infection of the injection holes across the chemical treatments was in range of 0 to 17 % (Table 12.4) with the exception of Treatments 4 (Arbotech, sodium hypophosphite and benzimidazole) and 12 (potassium salicylate), where infection rates were 46 and 83.3 %, respectively. Generally the injection treatments that had direct fungicidal activity had lower levels of infection as compared to indirectly acting treatments such as salicylic acid and potassium phosphite. However, the exception to this was Treatment 4 (Arbotech) that contained the fungicide benzimidazole, which is generally fungitoxic to Basidiomycetes but this was not observed in these studies. Arbotech was also found to be one of the less effective fungicides against *N. psidii* in earlier *in vitro* screening work (Table 12.1), which was surprising because this pathogen is sensitive to benzimidazole chemistries (such as benomyl), as also shown in Table 12.1. One possible explanation for the reduced fungitoxic effect of Arbotech may be due to the inclusion in the formulation of sodium hypophosphite, which is a very strong reducing agent (Rawcliffe and Rawson, 1969) and as such, it is a strong nucleophile in chemical reactions. Benzimidazole has a benzene ring fused to an imidazole ring. Both components of these structures are susceptible to nucleophilic substitution reactions (Carey and Sundberg, 1990) that can change the functionality of the molecule and hence possibly alter its toxicity to fungi.

Arbotech and potassium salicylate treatments induced the largest deadwood footprint in the

xylem tissues in terms of linear distribution (basipetally and acropetally), as well as the horizontal spread of tissue damage around the injection wound (Table 12.4). The strong reductive properties of sodium hypophosphite probably induced severe chemical shock to living tissue. Salicylates are SAR inducers, but these compounds can cause chemical tissue etching that form part of the basis of their ability to elicit the host response and as such they have the propensity to be phytotoxic (Ji and Wilson, 2003), particularly at the concentration of the material that was injected (10% v/v in water).

Previous studies on other tree vascular wilting diseases such as Dutch elm disease, indicated positive benefit in terms of disease control by injecting bacterial biological control organisms (Scheffer 1983; Scheffer and Elgersma, 1988; Scheffer, 1989). Specific species of *Pseudomonas* were found effective in suppressing *Ophiostoma ulmi* (Buisman) Melin and Nannf. (1934), the causal fungal pathogen of Dutch elm disease. The unsuccessful attempt at preventative biocontrol using *B. subtilis* Strain LC45 was not wholly unexpected when considering the challenges to success of this injectable treatment. The deployment of a foreign bacterium into the xylem vessel of a plant subjects the microbe to significant physiological stresses such as pH, osmotic shock and host defense chemicals. As such, attempts to establish biocontrol of vascular wilting disease have generally met with failure due to the significant obstacles in establishing the control agent in the vascular system of the host (Tsuda *et al.*, 2001). It was also interesting to note that all the injection holes resulting from the injection of the *B. subtilis* Strain LC45 injections were infected with *P. occidentalis*, indicating a complete lack of antagonism between the bacterium and the basidiomycete pathogen.

Polyporus occidentalis has not been previously reported infecting guava (or citrus) injection holes and a broader investigation as to the incidence of this fungus on citrus and avocado was initiated by the author (outside the scope of this current study). Citrus trees were also found to be susceptible to infection by *P. occidentalis* (Fig. 12.6). However, avocado was not susceptible. Although a hardwood by definition, avocado grows rapidly in its natural tropical habitat and exhibits a low wood density (wood density = $0.44g.cm^{-3}$), properties more closely resembling soft woods such as conifers [spruce = $0.431g.cm^{-3}$ (Seely, 2007)]. Guava has a high wood density [$0.67g.cm^{-3}$ (Lucus *et al*., 2006)], as does citrus [$0.59-0.7g.cm^{-3}$ (Brown, 1997)], densities which are typical of hardwoods. Avocado wood is very resilient to wood rots and it is postulated that this is in part due to the tree being a potent producer of anti-fungal dienes (Carmen *et al.,* 1998). In addition, on exposure to air, avocado wood undergoes an oxidative browning response, which is usually indicative of defensive phenolic metabolic activation (Bailey, 1973).

Fig. 12.6. Citrus injection holes infected with *P. occidentalis*. Mycelium emanating from a 2 year old injection hole (left). A fully developed basidiocarp emanating from a 2 year old injection wound (right).

Subsequent to these studies, limited trials were undertaken by the author (outside of the scope of this present study) to evaluate alternative sealants for sealing of the injection wounds. These sealants contained various biostatic compounds and/or sterilants. One product [(Bacseal®), Bayer Pty. Ltd., Isando, South Africa] is a paste that contains 10 g.kg^{-1} bitertanol plus 10 g.kg^{-1} 8-hydroxyquinoline sulfate. In preliminary tests it has exhibited high efficacy in significantly diminishing injection wound infection by *P. occidentalis* in both guava and citrus.

The chemotherapeutic agents tested here did not control GWD effectively, which is broadly in agreement with the studies by Leu and Kao (1979) in Taiwan. However in those studies, the author did not recommend the use of fungicides for post pruning protection, as suggested

378

herein. The fungicide treatments studied by Leu and Kao (1979) did not include the class of triazole fungicides, which in these studies (laboratory and field), as well as the *in vitro* studies of Joubert and Frean (1993), indicated the superiority of this fungicide class against *N. psidii*.

From these studies, the author would advocate the utilization of fungicides immediately after pruning as a precaution against aerial infection and part of an overall integrated GWD management program. Pruning protection with chemotherapeutic products and sanitizing agents has been widely adopted in many crops where fungal infection is associated with aerial canopy wounding (Spiers and Brewster, 1997; Sosnowski *et al*., 2008). In these studies, the application of these chemical and biological agents were applied in such a way to also coincide with the annual pruning schedule and hence designed to also afford protection against canopy infection.

In these studies, although impossible to separate out the specific impacts of these treatments on the aerial or subterranean infection pathways, it is interesting that the most effective "stand alone" chemistry deployed in these trials (propiconazole) has been found to be highly effective as a pruning wound protectant (Sosnowski *et al*., 2008), as well as having significant basipetal vascular distribution in trees (Amiri *et al.,* 2012). When propiconazole was combined with benomyl (an effective soil and locally systemic benzimidazole) and potassium phosphite (a systemic resistance inducing inorganic salt of phosphorous acid), GWD progression was inhibited to the greatest extent and resulted in the fewest tree deaths over the course of the 36 month trial period (Table 12.2). New strains of *N. psidii* have readily overcome new guava cultivars selected and introduced for superior GWD resistance, within a few seasons

(Schoeman and Labuschagne 2014). It is likely that this resistance is mostly monogenic, vertical resistance due to the rapidity of its breakdown in the field. As guava cultural practices are intensified, such as by the use of multiple pruning cycles, canopy pruning is now no longer confined to one cycle in the winter (when the pathogen is least active). Crop manipulation through pruning has necessitated earlier (in the life of the tree) and more frequent cycles conducted in the summer months (when the pathogen is most active), and as a consequence, greater selection opportunities for *N. psidii* mutations infecting new germplasm. With these new cultural practices emerging in guava cropping where there is now a confluence of greater pathogen activity and increased host potential infection courts, it seems prudent that the prophylactic use of fungicides are integrated into a GWD management programs.

Nalanthamala psidii is a wound pathogen, which in the hotter production regions, is particularly well adapted to the common pruning practices that occurs in the late winter/early spring months, when guava metabolism is at a low level, resulting in slower pruning wound healing and an expanded opportunity for infection (Chapter 7).

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382

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CHAPTER 13: SCREENING, SELECTION AND BREEDING GUAVA VARIETIES FOR RESISTANCE TO *NALANTHAMALA PSIDII,* THE CAUSAL ORGANISM OF GUAVA WILT DISEASE

ABSTRACT

Over several years, various approaches were undertaken to develop guava germplasm with improved resistance to *Nalanthamala psidii* (Biourge) Shroers comprising of: surveying guava regions of South Africa, Taiwan, Swaziland and Mozambique where guava wilt disease (GWD) is present; collecting potential escape trees from orchards and wild seedling plants; accessing guava germplasm previously selected for GWD tolerance (as in Taiwan); importing guava germplasm from guava producing countries; collecting seed from susceptible and resistant open pollinated orchards; challenging existing germplasm from repositories at the Institute for Tropical and Sub-Tropical Fruit Crops [comprising of imported varieties, tissue culture derived somaclonal mutations, mutagenized seedlings derived from gamma irradiated seed, as well as seedlings derived from open pollinated seed of South African varieties (cvs. Fan Retief and Malherbe)]. Large quantities of scarified seed were established in seed beds, grown to 30 cm in height, mechanically pruned, and finally inoculating the pruned plants with a mixed strain suspension of *N. psidii* conidia derived from Isolates CSF 221 and CSF t226. The process was repeated every 4 months. Survivors were removed and grown out for a further year. Clonal germplasm was asexually propagated to produce 1-2 year old containerized trees, as was undertaken for selected seedlings, for greenhouse trunk inoculation. Established orchard trees were initially challenged with *N. psidii* by inoculating pruning wounds. The survivors were trunk inoculated. Several selections that had remained disease free post-pruning wound inoculation did not survive trunk inoculations. Hence the latter method was considered a definitive challenge method to assess guava germplasm for GWD resistance. Field selections exhibiting GWD resistance were clonally propagated, established in the greenhouse and further challenged to confirm the putative resistance to GWD. Selected germplasm that survived multiple greenhouse challenges, were field planted into established GWD infected sites, in regional production areas in order to evaluate them for their disease resistance and crop characteristics. Several selections were derived from this program, including OT-1, a resistant derivative of the highly susceptible cultivar Fan Retief.

13.1 INTRODUCTION

Guava Wilt Disease (GWD) is the most destructive disease of guava and, as with most other vascular wilting diseases, it cannot be controlled curatively (Yadeta and Thomma, 2013). The long term goal in the development of any sustainable control program for vascular wilting diseases is the development of durable crop resistance (Pommer and Murakami, 2009). Throughout the climatic areas that can support the growth of guavas, wild seedlings are commonly found, spread primarily by birds and mammals and often found along road sides and bush areas close to commercial orchards. These wild guava seedlings are the bane of commercial growers because they serve as a reservoir for *Nalanthamala. psidii* (Biourge) Schroers, but, conversely, they also have the potential to be a source of naturally selected *N. psidii* tolerant germplasm. The ubiquitous distribution of 'wild' guava seedlings complicates disease management in commercial orchards. The problem is compounded by the sheer numbers of these wild seedlings, and consequently growers are reluctant to collectively invest capital for their removal. This resource, as well as a breeding program for the collection, screening and selection of *Psidium guajava* germplasm for resistance to *N. psidii* was initiated, as well as a rootstock field evaluation program. The aim of these studies was the identification of germplasm that had resistance to *N. psidii.* The development of quantitative resistance to *N. psidii* in guava cultivars is a pre requisite for a sustainable guava industry, given the inexorable global spread of this disease in the last several decades.

13.2 MATERIALS AND METHODS

13.2.1 Selection of Resistant Germplasm

Multiple strategies were employed over several years to obtain improved resistance to *N. psidii* in guava germplasm. Initially surveys in guava regions of South Africa, Taiwan, Swaziland and Mozambique, where guava wilt disease (GWD) is present was undertaken to identify and collect potential escape trees from orchards and wild seedling plants. Naturally occurring seedling selections that were in close proximity to the test site orchards or commercial orchards afflicted with GWD are potentially valuable sources of resistant germplasm. In addition, commercial orchards were inspected for escape tress or obvious bud sport mutations. Escape trees were identified, multiplied and monitored for durable resistance to *N. psidii* over several seasons. Promising escapes, seedling trees, seedling selections and imported germplasm, were vegetatively multiplied primarily by making shoot cuttings, to produce 1-2 year old containerized greenhouse trees which were further screened for *N. psidii* resistance by challenging these plants with *N. psidii* in the greenhouse utilizing stem inoculation methods as described in Chapters 2 and 3, comprising extracting a 5mm diameter plug of mycelium from 5 mm behind the advancing front of the expanding *N. psidii* colony on PDA. These plugs were inserted into a $2cm^2$ bark flap on the trunks, 10 cm above the soil line, of 1 year old clonal guavas (cv. Fan Retief). The wound was gently wrapped with Parafilm[®] (Pechiney Packaging Inc., New Jersey, USA, formally American National Can, Chicago, USA), followed by sealing with masking tape (3M, Minnesota, USA), as shown in Fig. 2.1 (Chapter 2). If seedlings expressed resistance to *N. psidii* they were challenged further by utilizing root and pruning wound inoculation. If they survived, they were evaluated for their horticultural traits (production and fruit quality) in commercial orchards afflicted with GWD.

Secondly, thousands of seeds were extracted from guavas collected from a seedling orchard

(Orchard 8) as well as from an orchard of Fan Retief trees (Orchard 7) and an orchard of Laia Pa trees (Orchard 6). These seeds were partially scarified by placing them in acidified (to pH 2.5 utilizing 40% w/w sulfuric acid) hot water (60°C°C) for 5 minutes to hasten the germination process. Approximately 500kg of fruit was processed from each orchard, the seed extracted and germinated in late spring/early summer in planting beds located in a lath house. Each bed was sown with approximately 250,000 seeds (7-8 kg). When the seedlings reached approximately 20 cm in height, each bed (1 x 5m) contained approximately 5-7000 seedlings. When these seedlings reached a height of approximately 30 cm, they were pruned back to approximately 20 cm in late spring/early summer using a motorized hedge cutter (the blade surface was surfaced sterilized with a 70 % w/w ethanol solution prior to use) and sprayed with a mixed strain (CSF 221 and CSF t226) conidial suspension of *N. psidii* (at approximately 1.25 x 10^6 spores ml^{-1}, to which 0.1 % Tween 20$^®$ was added), as described earlier in this thesis (Chapters 2 and 7). This process was repeated every 4 months and the plants were assessed for survivors at the end of the following summer. The most promising seedlings in terms of plant growth and disease resistance were then further challenged by trunk inoculation as described above. Survivors from this process were planted out into an orchard that was heavily infested with *N. psidii* (Orchard 7).

Thirdly, existing collections of germplasm at the ITSC, Friedenheim Research Station (located in Blocks A 3, 4, 5, 7, 9, 10 and 11) were challenged with *N. psidii* in the field by aerial and trunk inoculations, as described earlier in this thesis (Chapters 2 and 3). This germplasm was very genetically diverse and comprised of clones and seedlings (grown from the seed of various varieties) from South Africa, Australia, Zimbabwe, Swaziland, Mozambique, Taiwan and the

U.S.A. In addition, there were tissue culture derived somaclonal mutants as well as mutagenized, field grown seedlings derived from Fan Retief seeds that had been gamma irradiated (at 40 and 50 Gy units) by utilizing a Co^{60} radiation source located at the ITSC. These seedling trees ranged in age from 3 to 7 years old. These trees were, at the time of these experiments, not assigned any selection codes and at the onset of these experiments, the site was free of GWD.

Several conventional clonally propagated guava varieties were also located on the ITSC research station and are listed in Table 13.1. These varieties were challenged with *N. psidii* at least three times by a modification of the trunk inoculation method described earlier in this study. Trees were inoculated by inflicting an angled wound (approximately 30° to the vertical axis) in the lower main trunk) that penetrated the bark and woody tissues to approximately a depth of 1-2 cm. The wound was made with a 'panga' (a bladed African tool similar to a machete), that was surface sterilized between trees. A quarter section of a *N. psidii* colony on PDA that had fully colonized the agar surface, was excised and inserted face up onto the woody tissues beneath the bark flap on the tree trunks, at least 20-30 cm above the soil line (Fig. 13.1a-d). The wound was compressed by hand, wrapped with Parafilm® (Pechiney Packaging Inc., New Jersey, USA, formally American National Can, Chicago, USA), followed by sealing with masking tape (3M, Minnesota, USA). Trees were initially inoculated in spring and monitored over several seasons for the development of GWD. Surviving trees were identified and reinoculated as before, assessed for a further twelve months and any surviving trees were clonally propagated by air layering and established in the greenhouse. Varieties exhibiting resistance to *N. psidii* were identified and propagation material (2 node leaf cuttings and air

layers) was collected for further screening against *N. psidii*. In addition, fruit produced (if any) from trees that survived the first phase inoculations with *N. psidii*, were collected (10-15 kg) and the seed extracted. Seedlings emanating from these seeds were further challenged by conidial sprays to pruning wounds when they reached six months of age by utilizing the same technique as described above. Surviving seedlings were utilized for future breeding studies (outside the scope of this work) to further select durable resistance to *N. psidii.*

13.2.2 Rootstocks

Rootstocks identified previously (Chapter 9) as having the potential to provide resistant rootstocks were investigated. These were *Psidium cattleianum* Sabine (strawberry guava) and *Psidium friedrichsthalianum* (O. Berg.) Nied. (Costa Rica guava)*.* As reported in Chapter 9, Fan Retief was grafted on to these rootstocks and planted into an infested orchard (Orchard 2) and left unpruned. Five trees on each rootstock were planted out and assessed over three years for resistance to *N. psidii*. In addition, Fan Retief was grafted on to OT-1 and planted out as above. Clones of cultivars OT-1 and Fan Retief were also planted out as ungrafted trees at the same site as control plants.

13.3   DATA ANALYSES

Data were analyzed by one or two way analysis of variance and where appropriate, treatment means were separated using the Waller-Duncan *k*-ratio *t* test (Little and Hills 1990).

13.4 RESULTS

13.4.1 Selection of Resistant Germplasm

Trunk inoculations were effective in screening both young and mature trees (Fig. 13.1). Mature tree inoculations of selected varieties of guava showed that there were large differences in their susceptibility when challenged with *N. psidii* (Fig. 13.2); the guava germplasm expressing a broad range of responses to infection.

Generally selections and varieties screened against *N. psidii* were predominantly susceptible to the pathogen, but the South Africa varieties (Fan Retief, Malherbe, Madeira) were found to be the most susceptible to the pathogen. The partially resistant Taiwanese varieties [Laia Pai (low to moderate resistance) and Pei Pa (moderate resistance)] did eventually succumb to *N. psidii* at 30 and 42 weeks, respectively, as compared to Fan Retief, which succumbed at 18 weeks following trunk inoculations (Table 13.1). The cultivars Laia Pai and Pei Pa exhibited higher levels of resistance to pruning wound inoculations than to trunk inoculations.
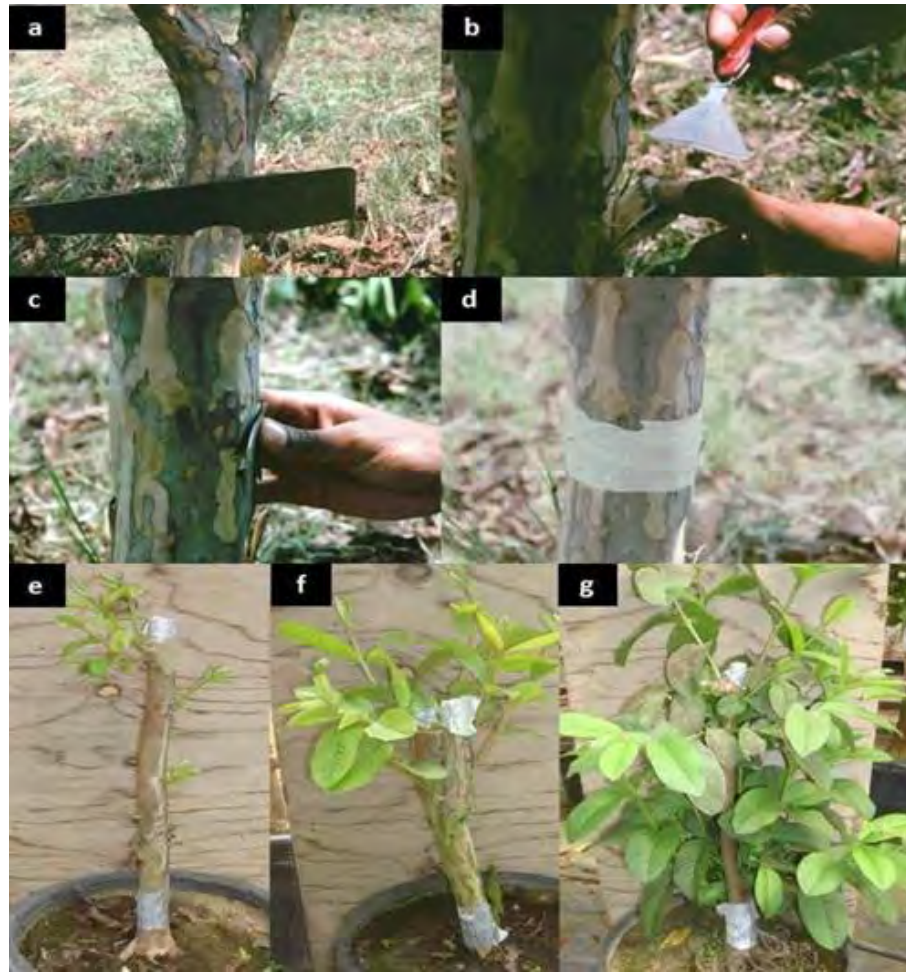
Figure 13.1. Final phase screening of resistant guava selections by main trunk inoculations.
a-d describe the main trunk inoculation process whereby a cut is made through the bark and into the wood, the flap opened and an actively growing agar culture of *N. psidii* inserted into the wound, after which it is sealed with Parafilm® and masking tape.
e - g, Off type selection one (OT-1) receiving a final aggressive bi-challenge with *N. psidii* by inoculating the main trunk just above the soil line and inoculating the cut surface of the main trunk. Wounds were sealed with Parafilm and tinfoil. e = 3 weeks after inoculation;
f = 5 weeks after inoculation; g = 8 weeks after inoculation.
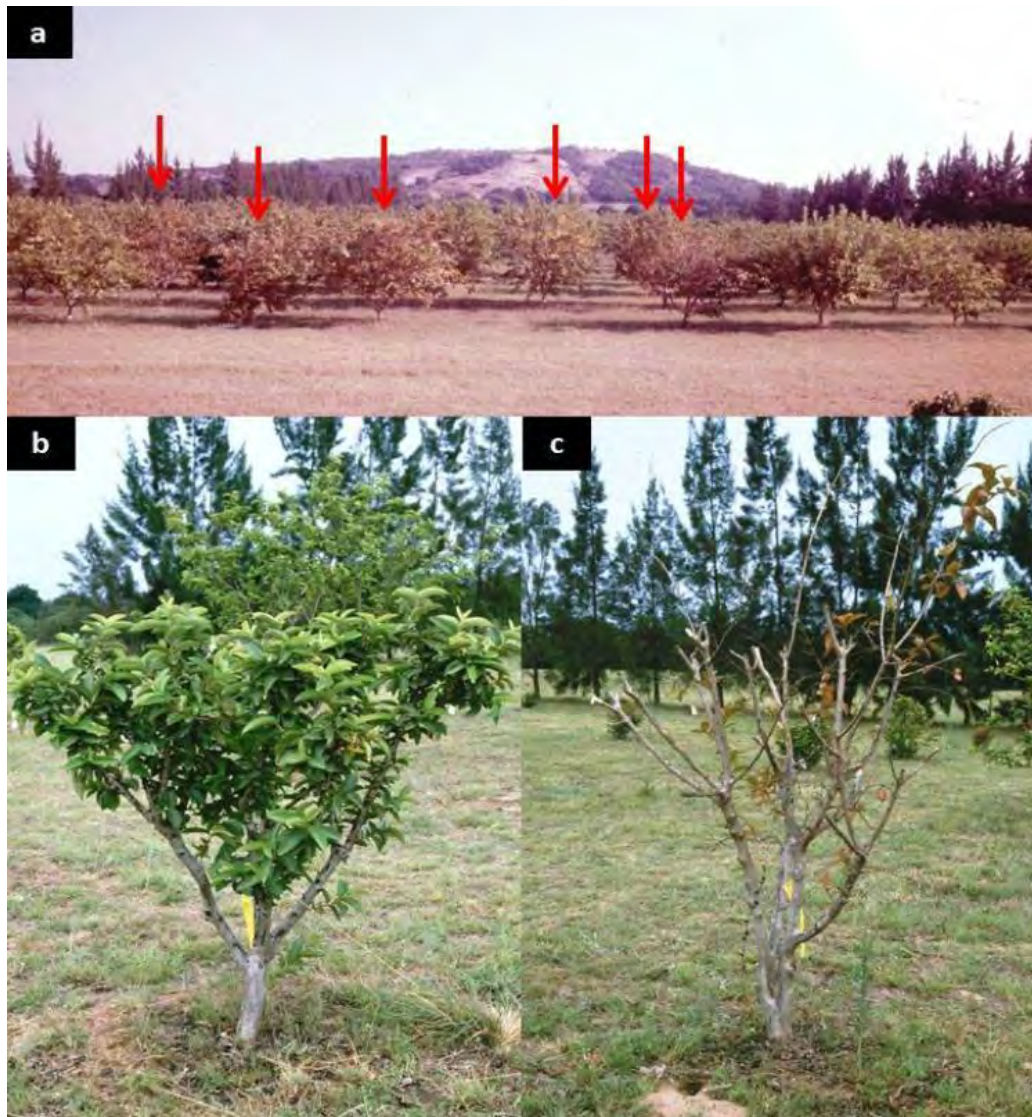
Figure 13.2. Open pollinated mature seedlings artificially infected with *N. psidii* by trunk inoculation.

a. Red arrows show diseased trees 4 months after inoculation.

b. Seedling guava tree inoculated with *N. psidii* by trunk inoculation, which previously was found tolerant to pruning wound inoculation.

c. The same tree three months later, close to death (stage 4 GWD rating scale).

Table 13.1. Susceptibility of mature commercial guava varieties to trunk inoculation with *N. psidii*

	Re-isolation[x]	Plant death[y]
Fan Retief	Y	Y (18)
Malherbe	Y	Y (16)
Saxon	Y	Y (21)
White Gower	Y	Y (27)
Madeira	Y	Y (18)
Anatola	Y	Y (20)
Thailand variety	Y	Y (27)
Beaumont	Y	Y (24)
Laia Pai	Y	Y (30)
Pei Pa	Y	Y (42)

All plants were between 3-7 years old.
A minimum of two replicates per variety were inoculated
[x] At plant death, woody tissue isolations were made from 10 cm above the wound site of each tree to detect the presence or absence of *N. psidii*.
[y] The figures in parentheses represent the mean time in weeks from inoculation to plant death. Y = yes.

Selection of open pollinated seedlings by multiple exposures to aerial inoculation of *N .psidii* was a moderately effective method to screen thousands of seedlings in a comparatively small area (Fig. 13.3).

Figure 13.3. Open pollinated seedlings being screened for resistance to *N. psidii* in the lath house.

a. Fan Retief open pollinated seedlings. The plants on the right are unpruned and the plants on the left are recently pruned and prepared for being treated with a foliar conidial spray of *N. psidii.*

b. Open pollinated seedlings of the Taiwanese variety 'Pei Pa' after one foliar challenge with *N. psidii*.

c. Guava seedlings 3 months after being challenged with *N. psidii*. Note the large number of plants that are dead in the central part of the seedling bed, indicative of uneven spore loading and/or growing conditions.

However, infection levels were not uniform in the seedling beds (Fig. 13.3), and disease levels

varied both within the season and between the season of application. The proportion of 1st

generation survivors was higher in the seedlings derived from the mildly resistant Taiwanese selection, Pei Pa, as compared to the progeny of Fan Retief (Fig. 13.4).

Time (months)

Seedling Source	T = 0	T = 12	T = 24	T = 36	T = 48
Fan Retief	26649	12105	5175	1031	256
Pei Pa	21678	14243	6445	2234	789
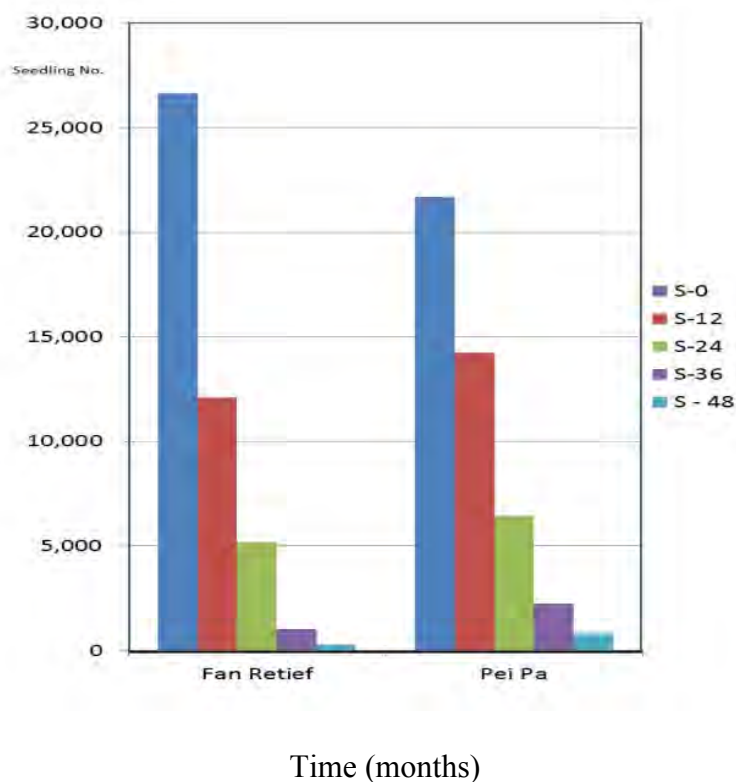
Figure 13.4. Open pollinated seedling survival after multiple challenges with foliar conidial sprays with *N. psidii*.
Fan Retief has little genetic resistance to *N. psidii* and hence the seedling survival rates after screening are much lower than with the Taiwanese variety Pei Pa, which has slightly greater resistance to the pathogen.
Values at T = 0 are the initial numbers of seedlings and subsequent values at T = 12/24/36/48 months, are the surviving seedlings, assessed in mid-summer following tri-annual aerial inoculations with *N. psidii*.

Seedling survival rates at the 24 and 48 month assessment following multiple aerial conidial inoculations were 19.4% and 0.96% for Fan Retief progeny, versus 29.7 and 3.6% for the mildly resistant Pei Pa (Fig. 13.4). Of the surviving seedlings at 48 months, approximately 150 of the healthiest and most vigorous plants were identified from both selections, and were further challenged by trunk inoculation. From the survivors, a total of 15 selections were identified that had resistance to *N. psidii*. These were clonally propagated and screened by trunk inoculation in the greenhouse. One selection was found to possess superior resistance, substantially better than that expressed by all other screened progeny of Fan Retief or Pei Pa when subjected to multiple trunk and canopy inoculations with *N. psidii*.

Above ground inoculations (particularly trunk inoculations), of mature field or large container planted, open pollinated guava seedlings were efficient in determining plant susceptibility to *N. psidii* (Fig. 13.1). Most susceptible trees showed GWD symptoms within two to three months, and most succumbed within four months of inoculation (Fig. 13.2). It was observed that some aerially inoculated mature guava seedlings exhibited sustained resistance (>18 months symptomless). However, when trunk inoculated, this level of resistance was not sustained (Fig. 13.5).

Figure 13.5. Open pollinated mature seedlings all artificially inoculated with *N. psidii* by trunk inoculation.
These three seedlings had previously withstood a foliar conidial spray of *N. psidii* applied to both green and brown tissue pruning wounds.
The yellow arrows indicate diseased trees.
The red arrow indicates a healthy tree.

Eight surviving mature open pollinated guava seedling selections that were found to survive four rounds of aerial inoculations (Table 13.2) were subjected to trunk inoculations. Only 3 plants survived this screening test. Clones of these plants were propagated and established in the greenhouse. The surviving orchard trees were maintained for future horticultural studies.

Table 13.2. Susceptibility of mature field established open pollinated guava seedling trees to trunk inoculation with *N. psidii*

	Post inoculation survivors after various time intervals[x]				
	Months				
	0	**12**	**24**	**36**	**48**
Tree number	1221	645	215	84	8

All plants were between 3-7 years old at the onset of the trial.
[x] All plants were pruning wound inoculated and any plants surviving 12 months were re-inoculated and assessed 12 months later.
This process was repeated 4 times over a period of 48 month.
Replication in this trial was not possible as all trees were seedlings.

A large number of wild trees were assessed over an eight year period for *N. psidii* resistance. With the exception of one tree, all were susceptible to *N. psidii* under controlled testing. The one escape tree identified as having resistance to *N. psidii*, was located just outside Orchard 2, along an access road and surrounded by diseased and dead trees. Fruit was collected and observed to compare favorably to Fan Retief for its horticultural traits. Clonal trees propagated from this tree were shown to have a very high and durable resistance to *N .psidii* in repeated challenges with both South African and Taiwanese isolates of *N. psidii* (Fig. 13.6).

Figure 13.6. A comparison of the Fan Retief guava cultivar to the resistant selection, 'Off Type one" (OT-1) in terms of resistance to trunk inoculation with the Taiwanese and South African strains of *N. psidii*.

a. From left to right; Fan Retief inoculated with the Taiwanese selection of *N. psidii*; Fan Retief inoculated with the South African selection of *N. psidii*; OT-1 inoculated with the Taiwanese selection of *N. psidii;* Uninoculated Control; OT-1 inoculated with the South African selection of *N. psidii*. Photograph taken 6 months after inoculation.

b. Close up of OT-1, 6 months after inoculation of OT-1 with *N. psidii*.

c. Fan Retief 6 months after inoculation with *N. psidii*.

Aggressive challenges with *N. psidii* utilizing trunk and severed main stem inoculations failed to induce tree mortality (Fig. 13.1) in this selection. Field plantings of this selection in *N. psidii* infected orchards also demonstrated high levels of disease resistance, acceptable fruit yield and quality (Fig. 13.7). Fruit harvest times were similar to Fan Retief. This selection was tentatively designated OT-1(Off Type 1).
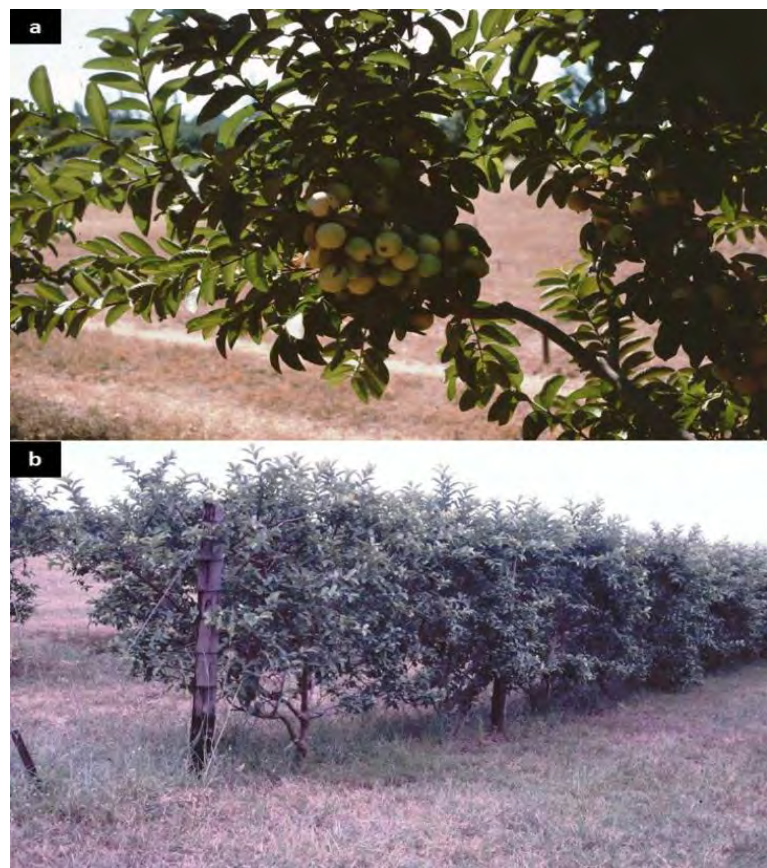
Figure 13.7. OT-1 being field tested in Orchard 2.
a. Fruit production.
b. A trellised hedge row of 3 year old OT-1 in a high disease pressure orchard.

13.4.2 Rootstocks

Rootstock screening trials with the *N. psidii* resistant *P. cattleianum* and *P. friedrichsthalianum* significantly increased survivability of Fan Retief as compared to when it was planted on its own roots (Table 13.3). At the end of the three year assessment, all the Fan Retief clonal trees were dead whereas all the other trees had survived, with the only exception being one Fan Retief tree grafted onto *Psidium cattleianum*. All the OT-1 trees survived, as did all the Fan Retief trees grafted onto OT-1 (Table 13.3).

Table 13.3. Susceptibility of selected guava varieties grafted onto guava rootstock selections as well as onto their own roots and planted into a heavily infested GWD orchard

	Trees surviving at [w]		
Scion/Rootstock	12 months	24 months	36 months
FR/PC	100	100	80 a
FR/PF	100	100	100 a
OT-1/PC	100	100	100 a
OT-1/PF	100	100	100 a
FR	40	0	0 b
OT-1	100	100	100 a
FR/OT-1	100	100	100 a

[w] Plants were annually assessed at the end of summer for 36 months from planting.
A minimum of five single tree replications per treatment.
FR = Fan Retief;
PC = *Psidium cattleianum*;
PF = *Psidium friedrichsthalianum*.
All trees were unpruned for the duration of the experimental period.
Figures followed by the same letter in each column do not differ from one another according to the Waller-Duncan *k*-ratio *t* test (P=0.05).

13.5 DISCUSSION

Previous studies (Chapters 2, 3 and 4) had shown that trunk inoculation of guava plants with

N. psidii to be an effective method of inoculating both young and mature trees. In these studies,

402

greenhouse, lath house and field inoculations of selected varieties, as well as open pollinated, mature seedlings, clearly identified the widespread susceptibility of most of the screened germplasm.

Guavas are diploids and the flowers are sexually perfect, bisexual and possess a degree of self-compatibility, reportedly in the range of 25-40% across most varieties (Morton, 1989). Additionally guava flowers have the ability to be pollinated by a wide range of insects. This is important as the prolific successful spread of the guava globally from its central American origin to all tropical and sub-tropical regions of the world has been dependent on the plant remaining productive without the need for specialized pollinators that it would have evolved with. Fortunately guavas have a floral structure that can be pollinated by a wide variety of insects and is highly attractive to many insect pollinators (Kwee and Chong, 1990). Genetic analysis has shown that between 26-43% of seed typically arise from cross pollinations, in various guava varieties (Soubihe and Gurgel, 1962; Kwee and Chong, 1990). As such, seedlings can possess large genetic diversity. Guavas also undergo anueploidic generation of triploids, a valuable trait that can reduce seed counts. Guava fruit development is less dependent on seeds for fruit development than many other fruit such as in the order, Rosales (Janick and Moore, 1996). The guava fruit can exhibit a significant range of parthenocarpy (Nagar and Raja Rao, 1983). These reproductive attributes of guava are desirable in the selection of improved varieties from open pollinated flowers, where out-crossing expands genetic diversity and concomitantly increases the probability of the creation of desirable traits (Janick and Moore, 1996) such as disease resistance.

Seed scarification assisted in condensing the seed germination and emergence period, which

can extend over eight weeks from the onset of germination until the completion of viable seed emergence (Kwee and Chong, 1990). As such, it was important to accelerate the germination period as far as possible to minimize the competitive effects of older plants on later emerging plants and thus increase stand density in the screening beds. Delayed emergence of guava seed may be exacerbated by reduced soil temperatures (Bhanuprakash, *et al*., 2008), and as such, seasonally extending the synchronized emergence of guava seed was a priority to maximize the productivity of these screening measures for resistance to *N. psidii*.

Initially the conidial aerial inoculation methods employed in selecting for *N. psidii* resistance resulted in many plant survivors, most of which were killed when trunk inoculated, utilizing agar cultures of *N. psidii*. Aerial inoculation in the seed beds produced non-uniform survival patterns (Fig. 13.3), which probably resulted from the uneven micro-climate in the beds, with the periphery experiencing much drier conditions than the central areas. As can be seen from Figure 13.3, the highest incidence of plant mortality occurred in the central part of the beds, whereas the survivor percentages were highest in the bed periphery. In addition, it was found that if artificial inoculation was attempted after too long a delay after pruning (approximately greater than 24 hr in winter and 48 hr in summer), infection success was greatly reduced. This result was broadly consistent with earlier findings (Grech, unpublished) that showed that receptivity of pruning wounds to *N. psidii*, was highly dependent on the age of the wound, the degree of host tissue suberization and the season (low temperatures reduced infection success). Other factors such as the tissue maturity (green or brown wood) and the consistency of the location of the pruning wound between challenged seedlings is important, as these, (unpublished) earlier studies by Grech, had indicated these factors can induce significant

variance in the host response to *N. psidii.*

In these studies, seasonality was an important source of variation in screening germplasm for resistance to *N. psidii.* In late spring, summer and early autumn aerial inoculations immediately following brown wood pruning resulted in more consistent infection than winter inoculations, where infection success was found to be lower and more inconsistent. In order to minimize variation and increase the reliability of this screening assay, procedures were developed to maximize seedling screening efficiency which generally entailed applying the *N. psidii* inocula to the targeted germplasm outside of the winter season. When these procedures were implemented in subsequent seedling screening assays, the seedling survival rates were consistent with previous work on the selection of *N. psidii* resistance in Taiwan by aerial inoculation (Wan and Leu, 1999). Wan and Leu (1999) reported survival rates in seedlings derived from controlled pollinations between parents with low to moderate resistance to *N. psidii* to be in the range of 0-48% after a six year screening program, with a mean survival value of 2%. The studies herein, produced survival rates after 48 months and multiple aerial inoculations, of 0.9% for progeny of Fan Retief (very susceptible) versus 3.6 % for progeny of the mildly resistant Pei Pa (Fig. 13.4). From these seedling selection trials, of the approximately 150 selections that were planted out in a diseased orchard, as well as being trunk inoculated in the greenhouse, 4 survivors were identified that had varying levels of resistance to *N. psidii* (Selections 2, 3, 4 and 6). One of these (Selection 3) exhibited the highest level of resistance (300 % greater than Fan Retief) and was horticulturally evaluated over several years for production and fruit quality marketability. Selection 3 was eventually released from the ITSC as the "Dimple" guava. Although it is more resistant than Fan Retief, it does not possess

satisfactorily durable resistance to *N. psidii*.

Aerial inoculation of guava seedlings is a useful tool that when applied under well-defined conditions, can be used to select germplasm resistant to *N. psidii* utilizing a comparatively small planting area. However, caution must be exercised in the over-reliance on this method to generate resistant selections because plants can show resistance to peripheral branch infection but may still be susceptible to major root or trunk infection, which appears to be governed by different resistance mechanisms. This was most evident in the guava germplasm field screening trials where some selections, which had withstood multiple pruning wound inoculations, succumbed to trunk inoculation. This observation was also evident in the pruning wound inoculations (green and brown wood) of mature trees derived from open pollinated seedlings, where aerial inoculation of pruning wounds did not always result in GWD development.

However, when these same trees were subjected to main trunk inoculation, most of these putatively resistant trees did not survive (Figs. 13.2 and 13.5). With a similar disease, Dutch elm

disease, infection courts are in the aerial parts of the tree, associated with insect feeding chambers. As in these studies, the most durable method assessed for screening germplasm of elm

for resistance was found to be trunk inoculations (Green *et al.*, 1984).

The Taiwanese selections Laia-Pa (low to moderate resistance to canopy infection with *N. psidii*) and Pei-Pa (moderate resistance to canopy infection with *N. psidii*) have been selected in Taiwan on the basis of these resistances to aerial infection. In these studies both succumbed

to *N. psidii* through trunk inoculation. These varieties are both white fleshed, targeting the Asia fresh market and hence they are horticulturally unsuitable for the South African processing industry, in which pink to red flesh is preferred. However, these varieties have more resistance to *N. psidii* than Fan Retief, and they were therefore utilized in these studies as a seedling and germplasm source for selection and breeding studies. It is interesting that the Taiwanese selections exhibited greater susceptibility when root or trunk inoculated as compared to their susceptibility to *N. psidii*, when inoculated through pruning wounds. Historically, Taiwanese guava breeding programs designed to select for resistance to *N. psidii*, have relied almost exclusively on the infectivity potential of pruning wounds as the basis for determining the relative susceptibility of germplasm (Leu and Kao, 1979). Recent studies in Taiwan have revised the importance of root and trunk infection in the etiology of GWD and established in that country the importance of rhizosphere transmission of *N. psidii* in guava orchards (Hong, *et al*., 2015). These studies have indicated that rhizosphere and root transmission of *N. psidii* is the major mode of transmission in mature guava orchards, in agreement with the findings reported herein.

Cell free filtrates derived from broth cultures have been used successfully to generate *N. psidii* resistant guava root stock selections, some of which have been commercially released in South Africa (Vos *et al.,* 2000). This method is not only cost effective but addresses the urgency of this catastrophic disease of guava, which drives the need for the urgent generation of germplasm that is resistant to this disease. Earlier studies (Grech, unpublished) identified potent plant wilting toxins in cell free culture filtrates from *N. psidii* broth cultures (Fig. 13.8).

Fig. 13.8. The effect of diluted (1×10^{-2}) axenic spent broth cell free culture filtrates from *N. psidii* on the greenhouse growth of guavas: a, soil application (right), control (left); b, foliar application (right), control (left); c, pre-emergent soil application to germinating guava seed (left), control (right). Note plant stunting in the cell free culture filtrate treatments.

This approach might be useful in providing an initial screening stage prior to inoculation with *N. psidii*.

Most of the resistant selections were unsuitable due their inferior yield and fruit quality. However, they could be utilized immediately as resistant rootstocks, an approach currently used in Brazil specifically to improve nematode management in guava plantations (Vasconcelos *et al*., 1997) and in India to improve wilt management in commercial orchards (

Bajpai *et al*., 2005; Kamle *et al*., 2012). Commercially acceptable varieties that are susceptible to *N. psidii* could be grafted onto resistant rootstocks. Grafted plants would then have significantly improved commercial survivability by avoiding root infection, which is a major mode of disease transmission of *N. psidii* (Grech, 1995; Hong *et at*, 2013 and 2015). Two potential *Psidium* species that have utility as rootstock and that may have promise after long term evaluation and graft compatibility studies are completed, are *Psidium cattleianum* and *P. friedrichsthalianum*. These two species when used as rootstocks, significantly increased the survivability of Fan Retief when grafted onto either of these selections (Table 13.3). However, both of these rootstock selections were derived from open pollinated seedlings and it was observed that Fan Retief grafted onto *P. cattleianum* and *P. friedrichsthalianum* plants exhibited significant variation. This variation manifested itself in terms of plant growth and graft union healing. Further selection of these two potential rootstocks varieties for use with commercial guava varieties will require further selection to ensure broad and durable scion-rootstock compatibility.

Rootstock utilization in fruit industries is widespread and utilized generally to overcome scionic production limitations such as root disease susceptibility (Hutchinson, 1985). In the utilization of rootstocks in combating GWD, if efficient sanitation during and immediately after pruning by the utilization of fungicides is implemented, it is likely that the infection opportunities for *N. psidii* would be substantially diminished.

Seed dispersal is efficient in guava biology due to mammalian and avian guava frugivory (Galetti *et al*., 2001; Berens *et al*., 2008). As such, guavas are found widely growing as weeds

usually in close proximity to commercial farms. This germplasm presents a significant opportunity to examine the genetic resistance of these seedlings, particularly if they are present in areas that have a high incidence of GWD. The collection and screening of 'escape' trees from areas heavily impacted by GWD was a significant component of the resistance screening studies herein. Additionally, trees were occasionally identified in orchards that seemingly were unaffected by GWD. Over several years and the screening of hundreds of escape trees for potential resistance to *N. psidii*, one selection was consistent in displaying a high level of resistance and having acceptable fruit qualities (Figs. 13.6 and 13.7). This selection was provisionally called "Off Type One" (OT-1) and was established in horticultural trials for longer term assessment of production and fruit quality. OT-1 was found to possess robust field resistance to *N. psidii* (Fig 13.7; Table 13.3) and is a valuable source of *N. psidii* resistant germplasm for future breeding efforts.

The management of guava wilt disease has proven to be particularly difficult due in part to the lack of effective resistance in South African commercial guava varieties, coupled to the relatively small current size (<1200 ha) and importance of the industry (Schoeman *et al.,* 2102). Chemical control has only succeeded in reducing the rate of disease induced tree mortality (Chapter 12). It appears to have a place in pruning wound protection, although even this step does not provide acceptable levels of protection, particularly with multiple annual pruning cycles. In the long term, continued plant breeding to develop fully resistant varieties (both rootstocks and scions) has to be viewed as an ongoing process by the guava industry. This has recently become apparent, where a *N. psidii* resistant selection identified as TS-G2 (developed by the ITSC, Nelspruit between 1995 and 2000) and that had been used as a scionic variety, as

well as a rootstock variety, was found in 2009 to have become vulnerable to *N. psidii* (Severn-Ellis *et al*., 2012). Schoeman and Labuschagne (2014) have further reported on this renewed epiphytotic affecting the Mpumalanga and Limpopo guava regions of South Africa, where *N. psidii* was found to be killing "resistant" guava varieties at a rate comparable to the susceptible Fan Retief cultivar. It became obvious from these studies that not only had the pathogen overcome the inherent resistance of these two ITSC cultivars, but also that two newly identified strains of the *N. psidii* pathogen (tentatively identified as G1 and G2) had emerged that putatively had developed enhanced virulence specifically towards the TS-G1 and TS-G2 guava selections. Interestingly, these two strains of *N. psidii* were less pathogenic on the Fan Retief variety, inducing only 20 and 40% mortality, respectively, when inoculated onto greenhouse grown Fan Retief plants relative to 100% mortality when the original Fan Retief *N. psidii* strain was used by the authors to root-inoculate plants. Some seedling selections (MS 44 specifically) exhibited moderately high levels of resistance to the G1 and G2 strains of *N. psidii* (50 and 78% survival, respectively, when root inoculated with the G1 and G2 strains under greenhouse conditions).

In these studies the *N. psidii* isolate CSF 221 was proven to be a virulent strain that exhibited high levels of pathogenicity against both Southern African guava clones and mildly resistant Taiwanese guava clones. Additionally, it caused high levels of mortality in genetically diverse, open-pollinated guava seedlings challenged with *N. psidii* by canopy inoculation. As reported earlier in this Chapter, survivors of these challenges frequently succumbed to trunk or major root inoculation.

The broad infective potential of *N. psidii* across wide spectra of germplasm also raises an

interesting opportunity in terms of the development of this pathogen as a biological herbicide, a field of research that has attracted much interest in recent years (Charudattan, 1991). In regions of the world where no commercial guava industry exists but where the plant has been inadvertently introduced and has become established as an invasive weed, this pathogen may have potential as a commercial bioherbicide, such as in Pacific and Caribbean islands. Both the common guava and the strawberry guava (*Psidium cattleianum*) are recognized as serious invasive weeds in these regions (Santos *et al*., 1989; Ellshof *et al*., 1995; Lowe *et al*., 2000).

Nalanthamala diospyri, a fungus closely related to *N. psidii*, has been used previously as a biocontrol agent of persimmon (Templeton and Greaves, 1984). In the 1940-50's artificially produced fungal inoculum of *N. diospyri* was distributed to farmers to control persimmon thickets in rangeland of central Oklahoma (Griffith, 1970). The agency distributing the inoculum (the Samuel Roberts Noble Foundation, Ardmore, Oklahoma) is still in existence today as a non-profit organization providing agricultural research and expertise primarily to the State of Oklahoma. Ranchers were provided with fresh spore suspensions and advised to inoculate the trunk 1 m above the ground because this was more efficient than inoculating higher up in the canopy. Wounding was generally inflicted with a hand axe and a spore suspension applied, which is remarkably consistent with the methodologies described herein, earlier in this chapter. These methods were reported to induce tree mortality to persimmon within 12 months (Templeton, 1987).

State approval for use of *N. diospyri* for persimmon control in the Oklahoma rangelands was granted in 1965, and a novel method of deployment was developed that involved coating the

spores onto shotgun pellets and firing these into dense thickets (Templeton 1987). The disease spread through root grafts, eventually killing the whole stand. However, currently there is no federal registration for this fungus as a biocontrol agent.

Obvious restraints to the widespread use of *N. psidii* as a bioherbicide include non-target impacts (such as spread to commercial orchards or other species in the Myrtle family that may be susceptible). The introduction of a fungal pathogen into countries where the pathogen has not previously been reported (e.g. the Americas) would also present considerable quarantine issues and would require a long and expensive regulatory process to be followed.

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THESIS OVERVIEW

The common guava (*Psidium guajava*) has become a global crop since it was first collected in Central America by European explorers in the 16th century and established in the Philippines. Today it is grown commercially on four continents and is the most significant fruiting genera in the Myrtaceae, a diverse family containing many important plant genera. The guava fruit is utilized fresh as well as in processed products such as juice and canned products. The Myrtaceae is divided into two subfamilies; Leptospermoideae and Myrtoideae, the latter of which contains *Psidium*. Guava, *Psidium guajava* L., is a fruit tree crop grown in the tropics and sub-tropics. It is a commercial crop in several countries such as India, Mexico, Brazil, South Africa, Philippines, Malaysia, Thailand, Pakistan and Taiwan. Wilting diseases of guava have afflicted this tree crop and continue to cause major economic losses in many of these production regions. *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans., has been reported as the causal pathogen of a widespread and destructive wilt of guava in India and Pakistan. Guava wilt disease (GWD) was first reported from Taiwan and is now widespread in Asia. It is caused by *Nalanthamala psidii* (Biourge) Schroers, and is now recognized as the most destructive disease of guava globally. This disease, once it is established in a guava production region, causes almost complete tree mortality, particularly where susceptible clonal plant production systems are in place. GWD has led to a substantial decline in viability of the guava industry in South Africa, which in turn has led to poor management of GWD in Southern Africa. Generally, fruits destined for industrial processing command a lower price than fresh fruit and therefore horticultural inputs tend to be minimal. As such, additional inputs costs and the uncertainty of future income as a result of GWD, often leads growers to exit guava cultivation and adopt an alternative crop system.

Guava wilt disease (GWD) causes major economic losses in South Africa (first reported in 1982) and Asia (first reported in 1926). GWD has been positively identified in Taiwan, Malaysia, Thailand, the Philippines, Indonesia and possibly in India. In Africa, GWD has been confirmed in South Africa, Mozambique, Zimbabwe and Swaziland. By 1987, approximately one million trees had died of this disease in these countries, where a highly susceptible clone (cv. Fan Retief) is the basis of guava production. The pathogen *N. psidii*, which up until a

revision of species in this genus by Schroers *et al.* in 2005, was comprised of obscure fungi largely unknown in culture.

Isolates of *N. psidii* from Asia and South Africa were equally infective when wound inoculated into guava, generally inducing death within 4 months. Isolates morphologically, physiologically and genetically were indistinguishable. This specialized pathogen was found in these studies to have unique etiological adaptations, such as: prolific conidiogenesis on moribund and/or dead host tissues of aerial and water disseminated propagules; two types of conidia with different optimal germination temperatures (Type 1, hydrophobic, produced on aerial tissues in sporodochia, wind dispersed, and Type 2, hydrophilic, produced on root tissue in sporodochia, water dispersed), both conidia equally infected on all tissues tested when wound inoculated; ability to produce chlamydospores; produce two morphologically distinct hyphae, a slender form that in host tissues was observed to breach xylem pits whereas the wider (approximately 3 times as wide) was observed to primarily colonize the xylem cavity in the longitudinal plane; endohyphae produced in culture and within the host's xylem tissues whereby the slender hyphae arise from septa within larger hyphae and progress through the degenerating wider hyphal structure (endohyphae) or from the cell wall and progress externally. Many of these morphological and physiological adaptations are reported here for the first time and represent evolutionary adaptions in this specialized pathogen of guava.

Pathogenically, *N. psidii* shares significant similarities to the persimmon wilt fungus and the palm pink rot fungus, both of which are now classified in *Nalanthamala* and described as *Nalanthamala diospyri* (Crandall) Schroers and *Nalanthamala vermoesenii* (Biourge) Schroers, respectively. *N. diospyri* induces a catastrophic wilt of the American persimmon that has resulted in the widespread elimination of this species from its native habitat of the South Eastern regions of the U.S.A.

Epidemiological studies in several orchards indicated that the disease primarily moved non-randomly in mature orchards, where root anastomosis was commonplace and which was intimated in tree to tree disease transmission. Tree mortality in summer generally exceeded 1.0 trees.ha^{-1}.day^{-1}, dropping to < 0.5 trees.ha^{-1}.day^{-1} in winter. These seasonal differences

are well correlated with the optimal temperatures for conidial germination and growth of *N. psidii*. Conidial trapping techniques deployed in diseased orchards, detected *N. psidii* propagules in both air and irrigation water.

Greenhouse and field inoculations, as well as observing natural infections of susceptible guava germplasm (cv. Fan Retief), indicated that from the onset of symptoms to tree death in field trees, generally required 2-3 weeks in summer and 4-5 weeks in winter. When greenhouse trees (cv. Fan Retief) were inoculated in their main trunk at 28°C, symptoms usually appeared within 10 weeks, followed by death after no longer than 16 weeks. When incubated closer to the pathogens optimal growth temperature (33°C), plants were usually dead by week 12. Pruning wound receptivity was similar at both inoculation temperatures, but it was discovered that the duration of receptivity was longer at lower temperatures probably as a result of slower wound annealing in this tropical plant. In areas affected by GWD, the winter dry season is usually when guavas are pruned. Although the pathogen at this time of year is less active, sporulation on dead tissue is profuse and hence the concomitant opportunity to infect pruning wounds that have a longer period of receptivity is a district possibility.

The moderately resistant Laia-Pai variety when trunk inoculated, expressed first symptoms at week 21 and died by week 37. Pruning wound inoculated Fan Retief greenhouse trees expressed symptoms within 1-2 weeks following inoculation but remained alive for on average 24-35 weeks, a period significantly longer than the trunk inoculated plants. None of the moderately resistant Laia-Pai trees that were pruning wound inoculated died. The discovery that the inoculation site had significant impact as to the expression of the degree of resistance in the guava selection is a critical finding in relation to the future selection of resistant germplasm. GWD symptom progression was characterized by field observation and controlled inoculations of susceptible (cv. Fan Retief) and partially resistant varieties (cv. Laia-Pai) and categorized into a disease rating scale comprising of 6 ratings and identified as the Guava Wilt Disease Rating Scale (GWDRS).

N. psidii was found to infect most guava tissue with catastrophic results, as the plant usually died shortly thereafter. Two tissues were identified where this was not the case; the fruit pedicel (fruit terminus) and the leaf. Leaf inoculation led to infection of the leaf tissue from which *N. psidii* could be recovered, but all infected leaves abscised prior to systemic plant colonization). This is the only known guava tissue that exhibits this hypersensitive response to *N. psidii* and in so doing averts plant death. The leaf inoculation technique developed in these studies is a useful method to initially screen host resistance as well as to screen *N. psidii* isolates for virulence without destroying whole plants.

Controlled studies investigating the histopathology of *N. psidii* in colonizing the active xylem (sapwood) tissues, indicated that the pathogen migrated acropetally more rapidly (2.8 mm.day^{-1}) than basipetally (0.9 mm.day^{-1}) and as such aerial infection of pruning wounds generally induced tree death over a longer period of time. Summer mortality was approximately 2-3 times faster than in winter and propagules were found to be viable in dead tissue for up to 12 months.

Rhizosphere inoculation of greenhouse and field guava (cv. Fan Retief) with *N. psidii*, resulted in plant death within 90-120 days. *N. vermoesenii* (Biourge) Schroers, inoculated into the rhizosphere prior to inoculation with *N. psidii,* did not induce any adverse effects on the guava, but it failed to protect against infection with *N. psidii*.

A critical finding in these studies was that mature (> 8 years old) guava orchards, in multiple locations, were found to have an exceptionally high degree of root anastomosis (> 70 % in the row and > 30 % between rows) and that these tissue grafts between major roots provide the primary mode of 'in orchard' transmission of GWD. Root transmission has recently been reappraised in Taiwan and their findings are aligned with those reported here, that root infection is an important and crucial disease pathway in commercial guava orchards. Previously in Taiwan, root infection was considered of much lower importance than aerial infection. These discoveries have profound implications for future control strategies of GWD and hence a reliable, rapid, non-invasive method to determine the degree of root grafting in guava orchards would be a useful management tool. The utilization in these studies of glyphosate to enumerate root grafting in commercial orchards was a novel approach and based on the property of this

fully systemic molecule to inhibit metabolic pathways primarily restricted to the root system. The technique proved to be useful and validated our hypothesis that basipetal migration of sub-lethal doses of glyphosate into the root system of the treated tree as well as the neighboring tree (if root grafts between the trees were present), would occur and be detected by the appearance of canopy herbicide damage. Future studies will be required to refine the technique, dosage, delivery and time of application, as in these experiments, excessive damage to the canopy occurred, whereas our aim was to induce minor leaf abnormalities, characteristic of chronic glyphosate residues in plant tissues.

Based on the information developed from epidemiological, histopathological and tissue infectivity studies, the construction of the first diagrammatic representation of the disease cycle of *N. psidii* on guava was undertaken. Further studies are required particularly to ascertain a clearer picture of the temporal dynamics of the rhizosphere phase of the life cycle of *N. psidii.*

The Myrtaceae is a family that contains many important fruiting genera, but economically the genus *Eucalyptus* contains the most valuable species, due to their prominence in the global timber industry. Prior to these studies, no broad assessment of the susceptibility of representative species within the Myrtle family had been undertaken whereby plants were challenged with *N. psidii* and subsequent re-isolation attempted. Critically, young seedlings (7 month old clones derived from cuttings), of several commercially important species within *Eucalyptus* were found to be susceptible. However this susceptibility was dramatically reduced when 2 year old clonal plants of the same species were challenged with *N. psidii,* and the two most important commercial species (*E. grandis* and *E. globulus*) were largely unaffected. Interestingly, *N. psidii* could be re-isolated from all the *Eucalyptus* inoculation wounds after 90 days. This is an interesting phenomenon and warrants further study in terms of the physiological activity level of the fungus whilst limited to the infection court tissues, what mechanisms keep the infection contained and whether there is a cogent propensity for the emergence of a pathogenic mutation to arise from these *N. psidii* colonies exposed to host tissue for prolonged periods. *Eucalyptus* is often grown in close proximity to guava, and these timber species are frequently coppiced, providing potential entry wounds for infection by *N. psidii,* from which the selection of an aggressive mutation is a possibility. This is an

area that requires further study due to the potential economic impact of such a scenario on the South African and global *Eucalyptus* based timber industries.

One species within the genus *Psidium* that was found to be susceptible beside *P. guajava*, was *P. montanum*. However, *P. cattleianum* and *P. friedrichsthalianum* were not only found to be completely resistant to *N. psidii*, but were also found to be graft compatible with the Fan Retief guava cultivar, which was investigated later in the thesis as to the suitability of these two *Psidium* species as rootstocks.

In guava orchards many anthropogenic abiotic wounding factors were identified (such as mechanical and hand weeding, desuckering, soil tillage and pruning). In addition, prominent biotic wounding factors active in all orchards surveyed in sub-tropical regions of Southern Africa, were phytophagous nematodes and carpenter moth larvae. Both inflict significant wounding in guava and reduce plant growth. It was hypothesized that these two organisms could inflict sufficient wounding to enable *N. psidii* ingress into the guava vascular system. It was determined that the role of nematodes in the etiology of GWD was not clear. A weak, transient synergy of the spiral nematode (at growth suppressing population levels) with *N. psidii* was discovered. Outside of a small delay in the onset of symptoms of GWD, nematode suppression by MeBr fumigation and/or nematode application did not reduce the velocity of GWD expression.

The metarbelid carpenter moth belonging to the genus *Salagena* was commonplace in guava orchards during the winter months. Two species (*S obsolescens* (Hanson) *and S. transversia* (Walker)) were endemic in all production region of Southern Africa. The larval stage of the salagena bark borer (SBB) was observed to induce multiple wounding sites in the bark and woody tissues of guava. Trunk, scaffold and secondary branches were most affected. It was postulated that as this insect is commonly found in guava orchards infected with *N. psidii,* and given that *N. psidii* is a wound pathogen, then SBB may be a disease synergist. In fact, damage of the tertiary branches caused by SBB and *N. psidii* can appear very similar (GWDRS, Categories 1-2). A method was developed to surface sterilize these insects with minimal mortality prior to their utilization in *N. psidii* inoculation studies. SBB was found in these

studies to exacerbate the development of GWD in the field and hence act as a synergist in the development of this disease. The presence of the SBB during the winter (dry) months, inflicting constant bark and wood damage of guava is particularly germane when devising management strategies for GWD. In winter, even though the pathogen is less active, tissue wounds (pruning wounds) were found in these studies to be susceptible to infection for longer periods than in summer, presumably due to more rapid healing at higher temperatures in this tropical species.

Very little is known about the interaction of adult carpenter moths and *N. psidii.* Can these insects convey conidia of the pathogen to new orchards? If so how far do these moths travel? A substantial knowledge gap is still required to fully understand the full role of these insects in the disease cycle of GWD. With the closely related fungus, *N. diospyri*, the causal fungus of persimmon wilt disease, Lepidopteran and Coleopteran bark borers have been clearly implicated in the wilt disease, and control of these insects is recommended as part of an overall management program to control persimmon wilt disease.

A strain of *Beauveria bassiana* (Bals.) Vuill., an entomopathogenic fungus, was discovered and was shown to be pathogenic on the SBB. It was also found to be an effective biological control agent for SBB in guava orchards when artificially grown and spray applied in infested orchards. The broader use of this organism to control SBB commercially has significant merit and is a research area that requires further investigation.

Methyl bromide (MeBr), although now largely banned as a soil fumigant, was used as a model fumigant to determine the impact on GWD and potential associated rhizosphere biotic synergists. It was postulated that due to the importance of the soil phase of *N. psidii* in the GWD cycle, elimination or a substantial reduction of the pathogen in the soil may reduce/retard disease progress. Soil fumigation was found to be effective in reducing the spiral nematode population to levels that did not impair tree growth. MeBr fumigation did retard the onset of GWD in treated plots and it did reduce the velocity of tree mortality in orchards. However, MeBr was not effective at eliminating *N. psidii* from infected root pieces with diameters larger that 1cm. Generally, the high susceptibility of the major commercial guava variety, the Fan Retief, coupled to the high dispersive potential of *N. psidii*, meant that in practice fumigation could not be relied upon to provide standalone control of GWD. However, the use of soil

fumigation to reduce resident soil inocula, combined with the use of GWD resistant guava varieties has potential and justifies further study.

Subsequent studies investigated the use of MeBr with chemotherapeutic and biological agents in order to further improve GWD management. Although the biological control treatments were ineffective in the field, some of the chemical agents reduced the progression of symptoms and the tree mortality rate. Specifically, propiconazole trunk injections, were the most effective singular treatment. Potassium phosphite injections, benomyl injections, foliar thiophanate methyl, and carbendazim foliar and soil drench treatments also reduced GWD progression and eventual tree mortality but at a lower level. Uniquely, propiconazole exhibited significant basipetal movement and a long residual activity in xylem tissues, which is postulated as being a major component of the suppressive effect of this treatment. A confounding factor was the post injection infection of trunk wounds by the mushroom *Polyporus occidentalis* Klotz [syn. *Coriolopsis occidentalis* (Klotzsch) Murrill] which was detected after several injection treatments. Koch's postulates were fulfilled and this white rot fungus was shown to be a pathogen that could inflict serious damage over the course of several seasons on the sap and heart wood of treated trees. Although several effective biocidal wound dressings were tentatively identified to treat injection wounds, expanded studies are required. Curative or prophylactic chemotherapy applied singularly or in combination with MeBr, were not effective in managing GWD. Prophylactic use of fungicides, coupled with improved durable resistance in commercial guava cultivars, might be a viable management option, particularly for the protection of wounds such as those arising during pruning. The final aspect of these thesis involved methods aimed at increasing the resistance in guava selections to *N. psidii*. This research started with surveys of guava production regions of South Africa, Taiwan, Swaziland and Mozambique where guava wilt disease (GWD) is present, in order to collect escape trees from orchards and wild seedling plants. Subsequently germplasm was accessed from other countries that produce guava, including guava varieties selected for GWD tolerance in other countries (as in Taiwan). Seed was also collected from susceptible and resistant open pollinated orchards. This collection of guava varieties was added to the collection of guava varieties held at the Institute for Tropical and Sub-Tropical Fruit Crops which included tissue culture derived somoclonal mutations, mutagenized seedlings derived from gamma irradiated seed. The entire

collection of guava varieties was then challenged with the pathogen, *N. psidii*. Challenge methods utilized two geographically diverse strains of *N. psidii* and varied methods of inoculation. A critical discovery was that some selections previously introduced into commercial production were tolerant to pruning wound infection but not to trunk inoculation, and hence the latter method was found to be the definitive challenge method. Initial screening programs required propagation and the generation of suitable quantity and quality of clonal source material prior to being challenged with *N. psidii*, a process requiring several years. Typically, the disease challenge process has a greenhouse stage of 3-4 years prior to planting out of the best selections to evaluate their horticultural performance in the field, which then requires several more years of observation. Several guava selections were also evaluated as rootstocks and in these studies, resistant rootstocks proved valuable in terms of improving orchard longevity. Several selections from these programs were identified that had durable resistance to *N. psidii*. Breeding strategies, selection programs and germplasm evaluation are key components of an integrated long term sustainable management plan for GWD.

This thesis has investigated and documented many aspects of *N. psidii* and the disease it causes, GWD, including macro- and micro-observations of the pathogen, confirmation of the taxonomy of the pathogen, the disease etiology, the disease epidemiology, the symptomatology of the disease and methods for the rating and control GWD. The research was field orientated and was conducted in many trials, over multiple seasons, in several geographic locations.

Some of the key outcomes of this research are: confirmation of the taxonomic status of this fungal pathogen as *N. psidii*; a clearer understanding of disease transmission in and between orchards; contributions to the promulgation of new legislation prohibiting the movement of plant material from the GWD infected regions of South Africa to the disease free guava growing regions of the Cape Province; the crucial role of root-to-root transmission of the pathogen between trees; the discovery that propagules of *N. psidii* can remain viable in dead woody tissues for up to 1 year; the discovery that *N. psidii* produces chlamydospores as well as extra-cellular plant wilting toxins; the discovery that the spread of GWD is exacerbated by certain pests, and by horticultural practices that are often applied in guava orchards; confirmation that currently available chemical control options are not effective; the discovery

of germplasm in the Myrtle family (including *Psidium*) that is tolerant of GWD, and which is graft compatible with guava; a finding that some non-*Psidium* species in the Myrtaceae are susceptible to GWD, but that the key timber genus, *Eucalyptus*, is generally resistant to *N. psidii*; and the identification of germplasm of guava with moderate to high resistant to *N. psidii* and acceptable horticultural qualities.

The seriousness and immediacy of GWD necessitated both a short and long term approach to the management of GWD. The studies undertaken in this thesis have contributed to the immediate management of GWD in terms of the validation of various integrated methodologies for commercially viable control of GWD such as soil fumigation, prophylactic and curative chemotherapy (particularly protection of pruning wounds), application of biocontrol agents, improved orchard sanitation, modification of cultural practices to avoid wounding (such as mechanical and manual weed control and desuckering), *in situ* burning of GWD killed trees and the restriction on the use of dead trees for firewood, soil ripping and severing root to root grafting between trees, application of certain soil amendments, modified fertility regimes, and irrigation sanitization treatments. The development of a 'best management program' for GWD, based on the studies herein and deployed in commercial orchards. In the longer term, the emergence of resistant germplasm from the multi-year screening programs set in place by the author is encouraging and vital for the sustainable commercial management of GWD.

An interesting aspect of the study of guava wilt disease is the potential for use of *N. psidii* as a biocontrol agent to control the common guava in parts of the world where it is considered to be as a noxious weed, such as in many of the Pacific islands. *N. diospyri* has been previously commercially distributed in various parts of the south eastern U.S.A. as a biocontrol agent for invasive persimmons in pastures.

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