Host plant resistance as a management tool for *Ditylenchus africanus* (Nematoda: Tylenchidae) on groundnut (*Arachis hypogaea*)

Sonia Steenkamp

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Promoter: **Prof AH Mc Donald**
Co-promoter: **Prof D de Waele**

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PREFACE

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ABSTRACT

HOST PLANT RESISTANCE AS A MANAGEMENT TOOL FOR *DITYLENHUS AFRICANUS* (NEMATODA: TYLENCHIDAE) ON GROUNDNUT (*ARACHIS HYPOGAEA*)

Groundnut is an important cash crop both for commercial and smallscale farmers in South Africa. The effect of *Ditylenchus africanus* on groundnut is mainly qualitative, leading to downgrading of groundnut consignments. This nematode is difficult to control because of its high reproduction and damage potential. The objective of the study was to investigate the potential of host-plant resistance as an effective and economically-feasible alternative management tool for the control of *D. africanus* on groundnut. Selected groundnut genotypes were evaluated against *D. africanus* in microplot and field trials. PC254K1 and CG7 were identified as resistant to *D. africanus.* The resistance expressed by these two genotypes is sustainable under field conditions. The resistance expressed by PC254K1 is effective even at high population densities. This genotype consistently produced yields with a low UBS % at all nematode population levels. PC254K1 could therefore be used as a major source of resistance to *D. africanus* in the development of commercial cultivars. Although the breeding line PC287K5 also maintained low nematode numbers in some trials, its level of resistance does not seem to be as strong or as sustainable as that of PC254K1 or CG7. However, PC287K5 could still play an important role in the groundnut industry where lower *D. africanus* populations occur. The resistance expressed by PC254K1 is not transferred to leaf callus tissue of this genotype, confirming there is no short-cut for screening for resistance to *D. africanus*. The reproduction and damage potential of *D. africanus* populations from different geographically-isolated localities in the groundnut-production areas of South Africa was tested under controlled and semi-controlled conditions and were found to be similar to each other. Resistance of PC254K1 to all of the tested populations was confirmed. These results indicate that the presence of this resistant trait in a cultivar developed from PC254K1 should be sustainable over the whole groundnut-production area of South Africa. The absence of *D. africanus* from pod tissue of PC254K1 confirmed the genotype's resistance. The mechanism of resistance involved may be the inhibition of proper development of this nematode, preventing it to build up to damaging population levels. However, PC254K1 is not immune to this nematode since it does occur in small numbers on this genotype. The resistance trait in PC254K1 is seemingly governed by a number of genes, implying that it will be

more durable under sustained pressure by *D. africanus* populations. Although markers associated with the resistance trait were mapped, they were not closely linked. Three putative qualitative trait loci (QTL's) were identified but markers associated with the resistance trait need to be refined and developed to be breeder-friendly in terms of marker-assisted selection. There are strong indications that CG7, which is a parent of PC254K1, may have more superior levels of resistance to *D. africanus* than PC254K1. The identification of markers closely associated with the resistance trait might, therefore, be more successful using CG7 in stead of PC254K1.

Key words: *Arachis hypogaea*, breeding, *Ditylenchus africanus*, groundnut, resistance, management.

UITTREKSEL

GASHEERPLANTWEERSTAND AS 'N BEHEERMATRIEEL VIR *DITYLENCHUS AFRICANUS* (NEMATODA: TYLENCHIDAE) OP GRONDBONE (*ARACHIS HYPOGAEA*)

Grondbone is 'n belangrike kontantgewas beide vir kommersiële en kleinboere in Suid-Afrika. Die effek van *Ditylenchus africanus* op grondbone is hoofsaaklik kwalitatief en veroorsaak dat grondboonbesendings afgegradeer word. Hierdie aalwurm is moeilik om te beheer weens sy hoë voortplantings- en skadepotensiaal. Die doel van hierdie studie was om die potensiaal van gasheerplantweerstand as 'n effektiewe, ekonomies-aanvaarbare alternatiewe beheermaatrieël vir die beheer van *D. africanus* op grondbone te ondersoek. Geselekteerde grondboongenotipes is teen *D. africanus* ge-evalueer in mikroplot- en veldproewe. PC254K1 en CG7 is geïdentifiseer om weerstandbiedend teen *D. africanus* te wees. Die weerstandseienskap van die twee genotipes is volhoubaar onder veldtoestande. Weerstand in PC254K1 is selfs effektief by hoë aalwurmbevolkingsvlakke en hierdie genotipe kan oeste met lae OGV % by alle bevolkingsvlakke produseer. PC254K1 kan dus as 'n hoofbron van weerstand teen *D. africanus* gebruik word om nuwe, kommersiële kultivars te ontwikkel. Alhoewel die teellyn PC287K5 in sommige proewe lae aalwurmvlakke kon handhaaf, lyk dit asof laasgenoemde se vlak van weerstand nie so sterk of volhoudbaar is soos dié van PC254K1 of CG7 nie. PC287K5 kan egter steeds 'n belangrike rol speel in die grondboonbedryf, veral in gebiede waar lae *D. africanus* besmettingsvlakke voorkom. Die weerstand wat in PC254K1 voorkom, word nie oorgedra na die blaarkallusweefsel nie, wat bevestig dat daar nie 'n kortpad is vir die evaluasie van weerstand teen *D. africanus* nie. Die voortplanting en skadepotensiaal van *D.* africanus bevolkings vanaf verskillende geografies-geisoleerde gebiede in die grondboon produksie area van Suid Afrika is onder beheerde en semi-beheerde toestande getoets. Die voortplanting en skadepotensiaal van die bevolkings was soortgelyk aan mekaar. Weerstand in PC254K1 is bevestig teen al die getoetste bevolkings wat daarop dui dat die weerstandbiedendheid van 'n kultivar wat uit PC254K1 geteel is, volhoubaar behoort te wees oor die hele grondboonproduksiegebied van Suid-Afrika. Die weerstand van PC254K1 word bevestig deur die afwesigheid van *D. africanus* in peulweefsel van die genotipe. Die meganisme van die weerstand is klaarblyklik dat die ontwikkeling van die aalwurm ge-inhibeer word en dus nie opbou na skadelike vlakke nie. PC254K1 is egter nie immuun teen dié aalwurm nie omdat hierdie parasiete nog in

klein hoeveelhede in die saad van hierdie genotipe gevind kan word. Die weerstandbiedende eienskap van PC254K1 word skynbaar deur 'n aantal gene beheer. Dit impliseer dat die weerstand meer volhoubaar is onder konstante bevolkingsdruk deur *D. africanus*. Merkers wat met die weerstandseienskap geassosieer is, is gekarteer. Hierdie merkers is egter nie nou gekoppel met die eienskap nie. Drie tentatiewe veelvuldige eienskaplokusse (VEL) is geïdentifiseer. Merkers geassosieer met die weerstandseienskap moet egter verfyn en verder ontwikkel word in telervriendelike merkers, wat in merkerondersteunde seleksie gebruik kan word. Daar is sterk aanduidings dat CG7 moontlik superieure weerstandsvlakke bo dié van PC254K1 mag hê. Eersgenoemde is 'n ouer van die laasgenoemde en as CG7 in die plek van PC254K1 gebruik word, mag merkeridentifikasie dalk meer suksesvol uitgevoer word.

Sleutelwoorde: *Arachis hypogaea*, beheer, *Ditylenchus africanus*, grondbone, teling, weerstand.

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CHAPTER 1

INTRODUCTION

1.1 THE GROUNDNUT CROP

1.1.1 Origin and distribution of the crop

The genus *Arachis* is native to South America and probably originated in central Brazil (Stalker & Moss, 1987) or northeast Paraguay (Simpson *et al.*, 2001). Wild *Arachis* species are distributed in a relatively small area that stretches between the Atlantic Ocean and the foothills of the Andes, from the mouth of the Amazon in the north to Uruguay in the south (Stalker & Moss, 1987). Gregory *et al.* (1973) suggested that most ancient *Arachis* species were found at high elevations. However, the geocarpic habit of *Arachis* suggests that long-distance dispersal occurred through water courses (Stalker & Moss, 1987) and that more recent speciation occurred as seeds were washed towards the sea (Gregory *et al.*, 1973). As the seeds dispersed to lower elevations they became isolated in major river valleys and different sections of the genus evolved in parallel evolution (Gregory *et al.*, 1973). The cultivated species *A. hypogaea* L. probably originated from the wild allotetraploid species *A. monticola* Krap. et Rig. since the latter is the only tetraploid known to be cross-compatible with *A. hypogaea* (Stalker & Moss, 1987).

1.1.2 Taxonomy of the crop

Arachis species fit into nine taxonomic sections including the cultivated groundnut *A. hypogaea*, which is subdivided into the subspecies *hypogaea* and *fastigiata* (Krapovicas & Gregory, 1994). Sub-species *hypogaea* includes the botanical varieties (var.) *hypogaea* (Virginia) and var. *hirsuta* (Peruvian runner) and subspecies *fastigiata* var. *fastigiata* (Valencia) and var. *vulgaris* (Spanish type) (Knauft & Wynne, 1995).

1.1.3 Cultivation of the crop

A. hypogaea is a self-pollinating, annual, herbaceous legume (Hammons, 1982) and is the only *Arachis* species cultivated extensively for commercial production of seed and oil (Stalker & Moss, 1987). *A. hypogaea* flowers aboveground but carries its

pods beneath the soil surface in which two to four kernels are formed per pod, depending on the variety and cultivar (Dickson & De Waele, 2005). Groundnut is cultivated over six continents (Dickson & De Waele, 2005) in a wide range of field conditions ranging from clays to sands and from acidic to alkaline soils (Stalker & Moss, 1987). Globally, approximately 20 million hectares are under groundnut cultivation (Carley & Fletcher, 1995) in approximately 100 countries with tropical, sub-tropical and warmer temperate climates (Naidu *et al.*, 1999).

Seventy two percent of the world's groundnut supply is produced in the Peoples' Republic of China, India, USA, Indonesia, Argentina, Senegal, Zaire and Myanmar (Dickson & De Waele, 2005). Global production averages nearly 24 million metric tonnes (Carley & Fletcher, 1995) and a significant proportion thereof is grown by resource-poor, smallholder farmers in developing countries (McDonald *et al.*, 1998). In Africa, approximately 5.3 million hectares are cultivated with groundnut (Carley & Fletcher, 1995) and major producers include Nigeria, Senegal, South Africa, Sudan, Zaire and the Democratic Republic of Congo (De Waele & Swanevelder, 2001).

1.1.4 Groundnut as a food source

Groundnut is listed as one of 20 crops standing between man and starvation (Wittwer, 1981). The calorie-rich kernels contain 25 % protein, 50 % oil, 20 % carbohydrate and 5 % fibre and ash (Knauft & Wynne, 1995) and may be boiled, broiled, roasted, fried, ground into butter, used as confectionary or crushed for oil (Dickson & De Waele, 2005). In the largest part of sub-Saharan Africa groundnut is an important subsistence crop grown mostly under rain-fed conditions (Van der Merwe *et al.*, 2001). Groundnut is primarily used as a cash crop and even smallholder farmers may sell their entire harvest (Stalker & Moss, 1987), which contributes significantly to food security and the alleviation of poverty in some countries and communities (Smartt, 1994). In South Africa groundnut is an important cash crop both for commercial and smallholder farmers (Mc Donald *et al.*, 2005).

1.1.5 Production constraints

Since groundnut is either a processed or a directly consumable food source, optimum kernel quality is important at all levels of production and utilisation (Hinds *et al.*, 1992; Swanevelder, 1997). Groundnut production and kernel quality can be limited by many abiotic and biotic factors, however. Drought is the most critical

abiotic factor that limits yield (Stalker, 1997). Yield constraints can also be caused by a calcium deficiency (Sumner *et al.*, 1988), phosphorus-deficient soils and a less-than-optimal relationship between nitrogen-fixing bacteria and the plant, inefficient nitrogen fixation due to environmental conditions (Knauft & Wynne, 1995), herbicidal and other chemical injuries or nutrient imbalances (Heagle, 1997). Iron, zinc, magnesium or boron deficiencies that occur in localised areas with a high pH also place constraints on groundnut production (Stalker, 1997; De Waele & Swanevelder, 2001). Biotic factors affecting yields include weeds (Knauft & Wynne, 1995), insects (Isleib *et al.*, 1994; De Waele & Swanevelder, 2001), diseases (Knauft *et al.*, 1988; Sharma & McDonald, 1990; Subrahmanyam *et al.*, 1990; Reddy, 1991; Mehan *et al.*, 1995; Murant *et al.*, 1995; McDonald *et al.*, 1998; De Waele & Swanevleder, 2001) and nematodes (Stalker & Moss, 1987; Kokalis-Burelle *et al.*, 1997; Dickson & De Waele, 2005).

Worldwide, plant-parasitic nematodes are primary parasites of groundnut (Dickson & De Waele, 2005) and are able to cause detrimental losses in groundnut production (Kokalis-Burelle *et al.*, 1997). New nematode species that cause crop damage continue to be discovered worldwide (Barker *et al.*, 1994) and may also be associated with high levels of aflatoxin and other soil-borne diseases (Porter *et al.*, 1982; Timper *et al.*, 2003). Annual losses caused by nematodes are globally estimated at 10 to 12 % when various crops are considered (Kahn, 2008), which translates into monetary losses of approximately US$6 billion in the USA alone (Agrios, 2005). However, the impact of plant-parasitic nematodes may be even higher than estimated since plant symptoms of nematode damage are usually nonspecific and yield losses caused by plant-parasitic nematodes often go unnoticed (Barker *et al.*, 1994).

Economic losses caused by nematodes will be much higher without the application of various nematode-management strategies and tactics (Barker *et al.*, 1994). In many regions of the world groundnut cannot be grown without the effective management of nematode populations (Porter *et al.*, 1982). Although many plant-parasitic nematodes have been associated with groundnut production locally (Venter *et al.*, 1992) nematodes were not considered to be a major pest until the discovery of the groundnut pod nematode, *Ditylenchus africanus* Wendt, Swart, Vrain and Webster, 1995 (Jones & De Waele, 1988; De Waele *et al.*, 1989).

1.2 THE GROUNDNUT POD NEMATODE

1.2.1 Origin and distribution

D. africanus was first discovered on severely damaged hulls and seeds of groundnut collected from a rain-fed field in the Schweizer-Renecke district (27.19°S, 25.33°E), North West Province, South Africa during May 1987 (Jones & De Waele, 1988; De Waele *et al.*, 1989). A national survey conducted during 1989 implies that this groundnut-parasitising nematode was present in the whole groundnut-producing area (De Waele *et al.*, 1989). Of the 877 seed samples collected during this survey that were graded as damaged, 73 % was infected with this nematode (De Waele *et al.*, 1989).

1.2.2 History

D. africanus was originally identified as *D. destructor* Thorne 1945 (Jones & De Waele, 1988), which is an important pest of potato tubers and flower bulbs in temperate regions of Europe, the USSR and localised areas in the USA (Hooper & Southey, 1982). A molecular study on the comparative taxonomy between some *Ditylenchus* populations (Wendt, 1992) and analysis of the ribosomal DNA (rDNA) of several geographic and host isolates of *D. dipsachi* Filipjev, 1936, *D. myceliophagus* Goodey, 1958 and *D. destructor* (Wendt *et al.*, 1993) however, casted doubt on the original classification. The local *Ditylenchus* associated with groundnut, furthermore, did not damage potato tubers (De Waele *et al.*, 1991) or other crops (De Waele *et al.*, 1989) and was consequently considered to belong to a different ecotype (De Waele & Wilken, 1990) that formed a distinct *D. destructor* race with a limited host range (De Waele *et al.*, 1991). Wendt and Webster (1992) also indicated that the rDNA of *D. destructor* specimens from South Africa differed from that of *D. destructor* specimens from the United Kingdom and Wisconsin, USA. Based on the characteristics of morphology and restriction fragment length polymorphisms (RFLP's) of ribosomal DNA (rDNA), *D. africanus* was finally described in 1995 as a new *Ditylenchus* species (Wendt *et al.*, 1995) that parasitizes various crops (Basson *et al.*, 1990) and weeds (De Waele *et al.*, 1990 & 1997) but causes damage only to groundnut (De Waele *et al.*, 1989). So far *D. africanus* has not yet been reported on groundnut from other parts of the world and it seems to be endemic to South Africa (Dickson & De Waele, 2005).

1.2.3 Life cycle and reproductive potential

Most of *D. africanus*' eggs (up to 13 eggs per female within 24 h) are produced at 28 °C and have a viability of 90 % (De Waele & Wilken, 1990). At 28 °C the life cycle from adult to adult can be completed within six to seven days (De Waele & Wilken, 1990). Greenhouse experiments indicated a 341-fold increase in numbers at harvesting (Venter *et al.*, 1991) and a 600-fold increase *in vitro* on groundnut callus tissue after only five weeks (Van der Walt & De Waele, 1989). Numerous generations can, therefore, be produced during a single growing season because of the short life cycle of *D. africanus* and favourable soil temperatures in the groundnut-production areas, which often exceed 25 °C at a depth of 0 to 30 cm (De Waele & Wilken, 1990).

1.2.4 Survival

In the absence of groundnut *D. africanus* can survive in low numbers on cotton, cowpea, dry bean, grain sorghum, lucerne, lupin, maize, pea, soybean, sunflower, tobacco and wheat (Basson *et al.*, 1990). The latter crops are commonly grown in South Africa and are often included with groundnut in crop rotation systems (Basson *et al.*, 1990). Weeds including cocklebur, feathertop chloris, goose grass, jimson weed, khaki weed, purple nutsedge and white goosefoot are commonly found in groundnut fields and can also serve as temporary hosts (De Waele *et al.*, 1990 & 1997). *D. africanus* can also survive South African winters for at least 28 to 32 weeks in hulls left behind in the field after harvesting (Basson *et al.*, 1992). Anhydrobiosis is one of the main survival strategies of this nematode (Jones & De Waele, 1990; Basson *et al.*, 1993), during which storage time of seed has no negative effect on surviving nematodes (Basson *et al.*, 1993). Nematodes that survived in hulls and seed can re-infest and damage a subsequent groundnut crop, even from small initial population densities (De Waele & Wilken, 1990; Venter *et al.*, 1991; Basson *et al.*, 1992; Mc Donald *et al.*, 2005).

1.2.5 Symptoms and histopathology

Symptoms of *D. africanus* resemble black pod rot caused by the fungus *Chalara elegans* in irrigated groundnut fields (Labuschagne *et al.*, 1980; Prinsloo, 1980) and are similar to those caused by *Aphelenchoides arachidis* (De Waele *et al.*, 1989),

which was reported on groundnut seed in Nigeria (Bos, 1977; Bridge *et al.*, 1977; Bridge & Hunt, 1985).

The initial symptom caused by *D. africanus* appears at the primary infection site located on the peg near the connection point at the base of the pod (De Waele *et al.*, 1989; Jones & De Waele, 1990) (Fig. 1.1).

Figure 1.1. Initial symptoms on groundnut pods caused by *Ditylenchus africanus* infection appears at the connection of the peg to the pod.

The outside tissue infected with *D. africanus* appear dark brown and corky and brown and necrotic on the inside upon removal of the peg (De Waele *et al.*, 1989). *D. africanus* usually penetrates the hull endocarp through openings at the base of the exocarp or at the pod apex (Jones & De Waele, 1990). Infected seed are usually shrunken, with dark brown to black micropyles and flaccid testae with darker vascular strands (De Waele *et al.*, 1989). The testae of infected seed can, furthermore, easily be removed by gentle rubbing and reveals a distinct yellow discoloration on its inner layer (De Waele *et al.*, 1989).

Histologically the feeding behaviour of *D. africanus* causes collapse, malformations and cell wall degradation (Venter *et al.*, 1995). This nematode feeds on the parenchyma cells surrounding vascular bundles just below the surface of a pod (Jones & De Waele, 1990). At advanced stages of the disease *D. africanus*-infected pods appear dead, with dark brown to black veins (De Waele *et al.*, 1989). Feeding of the nematodes near or in vascular bundles of the seed testa results in darkened

veins (Jones & De Waele, 1990). *D. africanus* does not penetrate the cotyledons but do feed on embryos (Jones & De Waele, 1990), causing them to turn olive-green to brown (De Waele *et al.*, 1989) (Fig. 1.2).

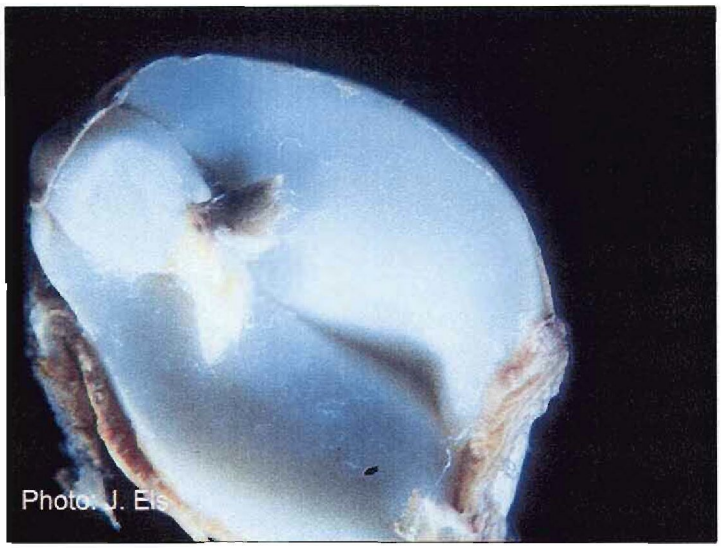

Figure 1.2. *Ditylenchus africanus* on the cotyledon and embryo of an infected groundnut kernel.

1.2.6 Damage potential and economic importance

D. africanus is considered to be one of the economically most important plant-parasites that limit groundnut production locally (Jones & De Waele, 1988; Venter *et al.*, 1991; Swanevelder, 1997) since this nematode causes severe losses in groundnut crops and income (Mc Donald *et al.*, 2005). At harvesting 90 % of a *D. africanus* population occurs in the pods, which consist of pegs, kernels and hulls (Basson *et al.*, 1991; Dickson & De Waele, 2005). Penetration of *D. africanus* at the infection site located on the peg near the basis of the pod (De Waele *et al.*, 1989; Jones & De Waele, 1990) causes weakening of the peg and pod connection so that pods break off during lifting of the crop (Fig. 1.3) and remains behind in the soil (Jones & De Waele, 1990). In heavily infested fields *D. africanus* might cause losses of 40 % to 60 % of pods in this way (Jones & De Waele, 1988).

Figure 1.3. Groundnut plants are lifted at harvesting. In severely-infested *Ditylenchus africanus* fields, 40 to 60 % of the pods breaks off and remains behind in the soil.

The main effect of *D. africanus* on groundnut is qualitative, however (Jones & De Waele, 1988 & 1990; De Waele *et al.*, 1989; Mc Donald *et al.*, 2005). Breakdown of the hull because of *D. africanus* damage increases water penetration into the pod (Venter *et al.*, 1995) and weakened pods often split open during severe infections (De Waele *et al.*, 1997). This breakdown of the hull and split pods result in the occurrence of second-generation seedlings (Venter *et al.*, 1995; De Waele *et al.*, 1997) (Fig. 1.4).

Figure 1.4. Groundnut seed and pods infected with *Ditylenchus africanus*.

Destruction of the seed testa caused by feeding of *D. africanus* (Jones & De Waele, 1990; Venter *et al.*, 1995), furthermore, leads to leaching of chemical compounds that function as inhibitors of seed germination (Svamv & Narasimhareddy, 1977) and result in the initiation of growth of the hypocotyls (De Waele *et al.*, 1997) (Fig. 1.4). Feeding of the nematodes near or in vascular bundles of the seed testa also results in an unattractive appearance of infected seed (Jones & De Waele, 1990) (Fig. 1.4).

These symptoms of *D. africanus* infections have a negative effect on the percentage of unsound, blemished and soiled (UBS %) kernels (Venter *et al.*, 1991; Van der Merwe & Joubert, 1992; Mc Donald *et al.*, 2005) and are highly correlated with the number of nematodes found in the testa of the groundnut seed (Venter *et al.*, 1991; Mc Donald *et al.*, 2005). *D. africanus* infestations, therefore, can have substantial financial implications for a producer (Van der Merwe & Joubert, 1992; Mc Donald *et al.*, 2005). Grading of groundnut consignments in South Africa is specified by law and kernels are classified into i) choice edible, ii) standard edible, iii) diverse or iv) crushing grade (Anon, 1997). Supply and demand dictate the prices of each grade and net gain increases with an increase in kernel grading (Mc Donald *et al.*, 2005). The economic importance of *D. africanus* is determined by the loss in income from infected groundnut consignments, which in turn depends on current prices for each grading class (Venter *et al.*, 1991).

1.3 NEMATODE MANAGEMENT STRATEGIES ON GROUNDNUT

Damage threshold levels are reached at the lowest nematode population density that is still able to cause a measurable reduction in plant growth or crop yield (Barker & Nusbaum, 1971). Control of plant-parasitic nematodes implies the application of a single measure to reduce or eliminate nematode pests, which in most cases is impossible (Viaene *et al.*, 2006). On the other hand, nematode management combines several different measures in consideration of the whole production system to achieve non-injurious or sub-economic threshold levels (Viaene *et al.*, 2006). Nematode management is considered effective when the nematode population remains below these damage threshold levels (Ferris, 1978).

As many current management options are becoming ineffective or unacceptable, new acceptable, environmentally-sound strategies must be developed (Barker *et al.*, 1994). The rationale behind nematode management is either food or profit and is, therefore, driven by resource availability. The ultimate goal of reducing nematode

numbers and increasing yield quantity and quality at cost-effective levels remain the same (Sikora *et al.*, 2005). In the 1990's nematode control measures included the use of integrated pest management systems (IPM) that still relied heavily on the use of chemical control (Sikora *et al.*, 2005). Globally the majority of land is cultivated by smallholder farmers using traditional methods (Altieri, 1984; Van der Merwe *et al.*, 2001). Because of economic constraints research, therefore, started to focus on low-input methods (Luc *et al.*, 2005). IPM developed into integrated crop management (ICM), concentrating on biological and cultural control methods (Sikora *et al.*, 2005; Viaene *et al.*, 2006) or natural pest management (NPM) strategies (Sikora *et al.*, 2005).

1.4 CURRENT NEMATODE MANAGEMENT TOOLS IMPLIMENTED FOR LOCAL GROUNDNUT PRODUCTION

1.4.1 Nematicides

Nematicides have been used since the late 19th century and continue to be an important part of nematode management programmes since their primary aim is to reduce the number of nematodes invading a crop while increasing yield quantity and yield quality (Haydock *et al.*, 2006). Although it is important for a nematicide to degrade into harmless compounds and not to persist in the environment, it is essential that their efficacy last long enough for efficient nematode control (Haydock *et al.*, 2006).

Nematicides currently registered locally for use on groundnut include fenamiphos and terbufos applied at planting and aldicarb, furfural and oxamyl applied at planting and / or at the onset of peg formation (Nel *et al.*, 2007). Nematicides are often used on groundnut to prevent damage to the pods later in the season (Sikora *et al.*, 2005) but long-term suppression of nematode populations is impossible to achieve with chemical control (Starr *et al.*, 2002). Nematicides are often ineffective in sufficiently reducing nematode population densities (Haydock *et al.*, 2006), especially those such as *D. africanus* which, because of its high reproduction rate produces more than one generation during a single growing season (De Waele & Wilken, 1990). Most of these nematicides currently registered on groundnut are, furthermore, effective for only eight weeks after application (Nel *et al.*, 2007), while the effective control of *D. africanus* requires a nematicide that remains active for at least 12 weeks after application (Basson *et al.*, 1992).

1.4.2 Cultural and biological management strategies

Nematicides are often too expensive for most smallholder farmers (Sikora *et al.*, 2005), therefore the latter rely on traditional methods for groundnut production (Van der Merwe *et al.*, 2001). For pest management programmes that exclude chemical control, cultural control methods are important alternatives (Viaene *et al.*, 2006). Cultural management includes the use of certified seed or nematode-free planting material (Viaene *et al.*, 2006). Local production of *D. africanus*-free, certified seed is hampered by factors such as the omnipresence of this nematode in the groundnut production areas (De Waele *et al.*, 1989), unpredictable efficacy of nematicides under harsh conditions (Mc Donald, 1998) and the unavailability of groundnut cultivars resistant to *D. africanus* (Basson *et al.*, 1991; Van der Merwe & Joubert, 1992; Venter *et al.*, 1993). Heat and mechanical methods of control are not suitable for the treatment of groundnut since its seed is soft, moisture-sensitive and easily damaged so that these treatments invariably affect germination (Swanevelder, 1997).

Biological control is another method that is an important alternative in a pest management programme that excludes chemical control (Viaene *et al.*, 2006). Although biological control holds some promise (Evans *et al.*, 1993), current knowledge on microflora and -fauna is not adequate for the successful establishment, promotion or effective suppression of nematode population densities, especially over the span of a single growing season (Starr *et al.*, 2002). Reliable and effective biological control systems are currently more likely to be limited to specialised situations where the environment could be manipulated in order to promote biological activity (Sikora *et al.*, 2005) and is, therefore, not adequate in keeping nematode populations below damage threshold levels on crops grown in most agricultural systems (Viaene *et al.*, 2006).

1.4.3 Crop-based management strategies

Crop-based management tools are mainly implemented to achieve high yields and improve soil fertility while reducing soil erosion, nematode, insect, disease and weed problems (Sikora *et al.*, 2005). Pest control through crop management includes starvation and trapping of the pest, antagonism and stimulation of soil antagonistic potential and / or biofumigation (Sikora *et al.*, 2005).

1.4.3.1 Crop rotation

Crop rotation remains one of the most important tools for nematode management (Viaene *et al.*, 2006). Well-planned rotation systems with other crops can aid in production of a high-quality groundnut yield (Swanevelder, 1997). Each production system has different requirements, however, and crops used in rotation are planted for different reasons (Sikora *et al.*, 2005). Locally effective management of *D. africanus* with crop rotation is hampered because of this nematode's ability to survive in small numbers on many crops other than groundnut, which are often used within rotation with groundnut (Basson *et al.*, 1990).

1.4.3.2 Resistant cultivars

The inclusion of resistant cultivars in pest management programmes is often preferred over chemical, biological, cultural or regulatory control components (Barker *et al.*, 1994) because host-plant resistance provides the most economical strategy for nematode management (Dickson & De Waele, 2005). Resistant cultivars provide additional benefits such as sustainability and cost effectiveness. They are environmentally benign (Cook & Starr, 2006), while effectively and economically managing nematodes on high- as well as low-value cash crops (Dickson & De Waele, 2005; Roberts, 2002) and imply little effort or additional cost to the producer (Starr *et al.*, 2002). Low-value crops that cannot support the costs of expensive pest-management inputs gain most from the planting of resistant cultivars (Fassuliotis, 1979). In developing countries and low-cash-crop systems, high-yielding, resistant cultivars are likely to be the only viable, long-term solution for nematode control (Roberts, 2002). To promote the sustainability of resistance, other management strategies should be combined with resistant cultivars (Sikora *et al.*, 2005), especially for those that do not express high levels of resistance or tolerance (Roberts, 2002).

Rotation with resistant plants is the most effective management tool for a number of *Meloidogyne* species parasitising groundnut (Nusbaum & Ferris, 1973; Barker, 1991; Rodriguez-Kabana, 1992; Noe, 1998). Groundnut sources expressing resistance to *M. hapla* (Castillo *et al.*, 1973; Subrahmanyam *et al.*, 1983), *M. javanica* (Sakhuja & Sethi, 1985), *M. arenaria* (Simpson & Starr, 2001; Simpson *et al.*, 2003) and *Pratylenchus brachyurus* (Smith *et al.*, 1978; Starr, 1984) are currently available. Rotation with resistant groundnut cultivars should, however, also be applicable for the control of *D. africanus* (De Waele *et al.*, 1990).

1.5 HOST PLANT RESPONSE

1.5.1 Host sensitivity

Host sensitivity depends on environmental effects, plant genotype and the number of parasites attacking a plant and is measured by the 'tolerant-intolerant' (or sensitive) continuum (Cook & Starr, 2006). Tolerant plants experience less yield suppression than intolerant plants (Cook & Evans, 1987; Trudgill, 1991; Roberts, 2002). Plants expressing extreme tolerance often show no symptoms of infection and are able to produce a normal yield (Bos & Parlevliet, 1995). However, tolerant plants usually have larger, healthier root systems and tend to allow greater nematode population increases (Roberts, 2002).

In contrast to tolerant plants, sensitive plants often have a smaller root system due to nematode injury (McSorley, 1998) and they usually react with relatively severe symptom expression e.g. including yield reduction (Bos & Parlevliet, 1995). Smaller root systems increase competition among parasites for feeding sites and food reserves, which causes a decline in the rate of population increase (Ferris, 1985; McSorley, 1998). A hypersensitive plant reacts violently to attacking parasites by preventing further spread of infection through prompt death of invaded tissue (Bos & Parlevliet, 1995). In some plant-nematode interactions resistance and tolerance are under separate genetic control (Evans & Haydock, 1990; Trudgill, 1991) and are often inherited independently from each other (Trudgill, 1991).

1.5.2 Host efficiency

Host efficiency is determined by the genetic interaction between plant and nematode, which is measured by the phenotypic continuum of 'susceptible-resistant' (Cook & Starr, 2006). With the exception of high temperatures that may sometimes erode the effectiveness of resistance mechanisms, environmental conditions often play a lesser role in the expression of host efficiency (Cook & Starr, 2006). Nematode densities expressed as number of nematodes per unit available host tissue is the main factor affecting host efficiency (Cook & Starr, 2006). A susceptible plant cannot impede the growth or development of a parasite (Bos & Parlevliet, 1995), which results in large increases in parasite populations, even from low initial densities (McSorley, 1998). A resistant host plant on the other hand resists penetration, development, reproduction and spread of a parasite (Bos & Parlevliet, 1995). The expression of resistance by a

host plant, therefore, depends on the plant's ability to interfere with the reproduction potential of the parasite (Sikora *et al.*, 2005) and can be described as low, moderate or high (reproduction of a parasite is only allowed at trace amounts or does not occur at all) (Roberts, 2002).

A plant is highly resistant when the final population densities (Pf) of a parasite are consistently lower than its initial densities (Pi) (Roberts & May, 1986; Windham & Williams, 1988) and when the reproduction rate of the parasite is lower than 10 % of the reproduction rate on a known susceptible reference (Hussey & Janssen, 2002; Timper *et al.*, 2003).

1.5.3 Genes expressing resistance

Resistance to a plant-parasite could be expressed by a single gene (monogenic), a few genes (oligogenic) or many genes (polygenic) (Roberts, 2002). Classification of genes is based on their phenotypic expression and includes major (large effects) or minor genes (small effects) (Roberts, 2002). Simple-inherited, major-gene resistance is often preferred because it is easier to identify and to incorporate in back-crossings or pedigree programmes using conventional breeding techniques (Roberts, 2002; Simmonds, 1991).

Resistance is classified as vertical (qualitative e.g. race-, pathotype- or biotype-specific) or horizontal (quantitative e.g. effective against all variants of the pathogen) (Van der Plank, 1978). Vertical resistance is controlled by one to three genes while horizontal resistance is polygenetically inherited as several minor genes, often with an additive effect (Roberts, 2002). The number of genes and their additive effects will determine the level of resistance expression (Jones, 1985). Horizontal resistance tends to be more durable or is less circumvented as a result of selection pressure on a nematode population (Roberts, 2002).

Expression of resistance is affected by i) genetic constitution of the host plant and parasite, ii) environmental effects and iii) virulence status of the nematodes (Roberts, 2002). Benefits of resistance are best demonstrated in moderately or severely infested fields since susceptible cultivars often express a higher yield potential if the nematode populations are below damage threshold levels (Sikora *et al.*, 2005). Apparent negative effects of resistance on yield are probably due to linkage drag whereby genes with negative effects on yield are linked to resistance loci (Cook &

Starr, 2006). However, no data exist to confirm a direct effect of resistance genes on reduced yields (Cook & Starr, 2006). Modern breeding programmes dealing with introgression of resistance usually make conscious efforts to increase the yield potential of resistant cultivars (Church *et al.*, 2005; Ogallo *et al.*, 1999).

1.6 MOLECULAR MARKERS

The regions within genomes containing genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs) (Collard *et al.*, 2005). Linkage maps are constructed using DNA markers to identify chromosomal regions containing genes that control simple and quantitative traits using QTL analysis (Collard *et al.*, 2005). DNA markers tightly linked to important genes may serve as molecular tools for marker-assisted selection (MAS) in breeding programmes (Ribaut & Hoisington, 1998). A locus consists of genetic markers occupying specific genomic positions within chromosomes and the development of DNA or molecular markers create opportunities to select for QTLs (Collard *et al.*, 2005).

Use of MAS together with phenotypic selection is more effective, reliable and cost-effective than conventional plant breeding methodology and is widely accepted as a valuable tool for the improvement of crops (Collard *et al.*, 2005). Genetic markers associated with *M. incognita* race 2 (Fourie *et al.*, 2008) and *M. arenaria* (Tamulonis *et al.*, 1997) resistance were identified in soybean for use in MAS and random amplified polymorphic DNA (RAPD) analysis resulted in the discovery of three markers associated with root-knot nematode resistance in groundnut (Burow *et al.*, 1996). RAPD is a high-volume technique in which multiple markers can be generated from a single DNA preparation (Collard *et al.*, 2005). Church *et al.* (2001) identified the RFLP markers R2430E and S1018E that flanked a dominant-gene locus for root-knot nematode resistance in groundnut. Although biotechnology is transforming ways in which resistance can be incorporated by the use of MAS, it does not eliminate the need for verification of the resistant phenotype by direct evaluation of nematode-host interaction in the field (Cook & Starr, 2006).

1.7 RATIONALE AND LAYOUT OF THE CURRENT STUDY

D. africanus is present throughout the local groundnut production area (De Waele *et al.*, 1989) and causes severe losses in groundnut crops and income (Jones & De Waele, 1988; Venter *et al.*, 1991; Swanevelder, 1997; Mc Donald *et al.*, 2005). Other

nematode species have been successfully and economically controlled through the use of resistant cultivars (Nusbaum & Ferris, 1973; Barker, 1991; Rodriguez-Kabana, 1992; Noe, 1998; Roberts, 2002; Starr *et al.*, 2002; Dickson & De Waele, 2005; Cook & Starr, 2006). Use of resistant groundnut cultivars may also be applicable for the control of *D. africanus* on groundnut (De Waele *et al.*, 1990). However, no *D. africanus*-resistant groundnut cultivars are currently available on the market. The objectives for this study, therefore, were to:

i) Identify at least one groundnut genotype with sufficient resistance to *D. africanus* that would also be sustainable under field conditions.
ii) Compare the reproductive and damage threshold levels of *D. africanus* on susceptible, tolerant and resistant genotypes.
iii) Establish whether the resistance expressed is present in callus tissue of this genotype.
iv) Establish whether there are differences in the reproduction and damage potential of *D. africanus* from different localities in the groundnut-production areas of South Africa on resistant genotypes identified in this study.
v) Establish the mechanism of resistance to *D. africanus* by means of histopathlogy.
vi) Establish the origin of the resistance trait.
vii) Identify possible molecular markers associated with the resistance trait.

To achieve the objectives set for this study, the chapters of this thesis consisted of the following:

Chapter 1 provides an overview on the groundnut crop and on *D. africanus* as a plant-parasite on groundnut in South Africa. Aspects discussed include the importance of groundnut as a food and income source and the qualitative effects of *D. africanus* on groundnut production. Management tools currently applied or available for *D. africanus* control are also discussed.

General materials and methods are provided in Chapter 2. Only those specific to each chapter were excluded in the latter and provided within the respective chapters.

Chapters 3 to 6 comprise the investigations done by the author.

Chapter 3 consists of three parts. In the first part of the study groundnut genotypes with *D. africanus* resistance were identified and the resistance was verified for sustainability under field conditions. In the second part of Chapter 3 the reproduction rate and damage threshold levels of a range of initial *D. africanus* population densities (Pi) were determined on Sellie, Kwarts, PC254K1 and PC287K5. In the third part of Chapter 3 the reproduction rate of *D. africanus* was studied on callus tissue initiated from leaves of Sellie, Kwarts, PC254K1 and PC287K5 to determine whether the resistance expressed by PC254K1 will be present in callus tissue.

Chapter 4 consists of comparative studies done on the reproduction and damage potential of five geographically-isolated *D. africanus* populations representative of the groundnut-production areas in South Africa. These studies were done under controlled and semi-controlled conditions in growth cabinets, greenhouse and microplots to determine differences in reproduction rates and / or temperature preferences and to compare the reproduction rates and damage potential of the five *D. africanus* populations on Sellie and PC254K1.

In Chapter 5 the mechanism of resistance to *D. africanus* expressed in PC254K1 was studied to determine histopathological differences associated with PC254K1's resistance.

Chapter 6 comprises a study on the genetics of the resistance identified in Chapter 3 and a search for molecular markers associated with the resistance trait. The number of gene(s) involved in the expression of the resistance trait was determined. Molecular markers were mapped and the magnitude of the association between the marker and *D. africanus* resistance was measured. Linkage analysis and drawing of the linkage map was done.

CHAPTER 2

MATERIALS AND METHODS

2.1 MICROPLOT FACILITIES

Microplot trials were conducted in two facilities located on the premises of the Agricultural Research Council – Grain Crops Institute (ARC–GCI). Contrary to greenhouse conditions, microplots allow for studies of soil-borne pathogens, their host plants, abiotic and biotic interactions under semi-controlled environmental conditions, i.e. conditions that are more representative of natural environments (Abawi & Mai, 1980; Johnson *et al.*, 1981; Caswell *et al.*, 1985). Microplots provide the additional benefit of the ability to manipulate the substrate as well as certain variables such as the introduction of specific nematode species and / or numbers (Johnson *et al.*, 1981).

One microplot facility used in this study (Fig. 2.1) consists of a set of 20 1.1 x 2.1 x 0.5 m^3 rectangular, clay-brick troughs. The troughs are built over a drainage system to prevent water logging. Plants are protected against hail by a hail net installed 2 m above the plots.

Figure 2.1. A microplot facility of 20 rectangular brick troughs covered with a hail net.

An irrigation system feeds two evenly-spaced micro-sprayers placed in the centre of each plot from a main feeding line, delivering approximately 25 ± 4 mm water in 15 min.

The other microplot facility (Fig. 2.2) consists of concrete pipes buried vertically in 15 rows. One row consists of eight, evenly-spaced pipes, each 500 mm deep with a diameter of 1 m.

Figure 2.2. A microplot facility of evenly-spaced, concrete pipes and hail-net cover.

The facility is also built over a drainage system to prevent water logging and a hail net, installed ± 3 m above the plots, protects plants from hail damage. The irrigation system feeding this facility consists of micro-sprayer lines connected to a main feeding line. Each micro-line feeds a pot through a micro-sprayer placed in the centre of the pot and delivers approximately 25 ± 4 mm water in 15 min. Irrigation of trials conducted in both microplot facilities was supplementary to rainfall.

2.2 SOIL FOR GREENHOUSE AND MICROPLOT TRIALS

A sandy-loam soil (Hutton) consisting of 93.6 % sand, 3.9 % clay, 1.9 % silt and 0.6 % organic material was used in the microplot and glasshouse trials throughout the study. The soil was obtained from a farm that is situated 24 km from Leeudoringstad (27.26°S, 26.47°E).

2.2.1 Fumigation of soil

Soil was fumigated after collection with ethyl dibromide AL (EDB) before filling of the microplots or plastic pots used in the greenhouse. EDB used during the current study is a registered nematicide and has an active ingredient of 1 800 g per l (Nel *et al.*, 2007). The product was used throughout this study at a rate equivalent to 50 l per hectare. Soil fumigation with EDB eliminates undesired organisms that could affect the data. EDB was applied manually with a special commercial hand applicator (Marunata, Telex 5423339, Marnak J., Kyoto, Japan) that is shown in Figure 2.3. The applicator consists of a sealable reservoir, injector handle and dosage control screw mounted on one end of a steel shaft. Situated on the opposite end of the shaft is an adjustable ring for depth control and release holes at the tip.

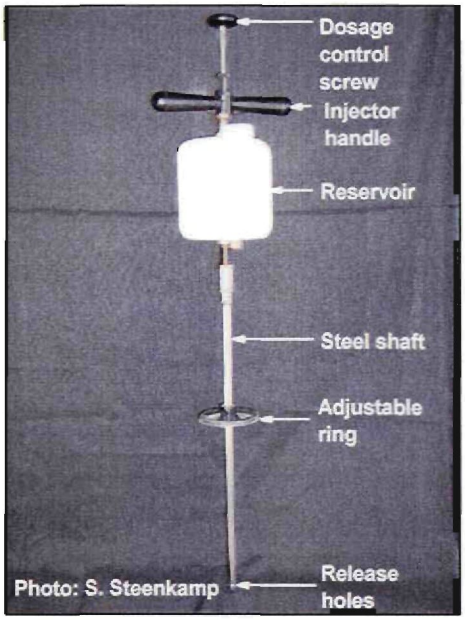

Figure 2.3. A special hand applicator used for EDB fumigation of soil.

Operators wore protective clothing and full-face gas masks during calibration of the applicator as well as during application of EDB to the soil. Accurate calibration of the applicator is achieved through adjustment of the control screw until the desired volume is obtained by each of 10 consecutive injections into a calibrated glass measuring cylinder. The required application depth was achieved and maintained by adjusting the adjustable ring to the required distance (30 cm) from the tip of the shaft.

Fumigation of soil for filling microplots or pots was conducted outdoors on a flat-surfaced area covered with a tarpaulin. Soil destined for fumigation was shovelled onto the spread tarpaulin in a layer of at least 30 cm deep to accommodate the part of the steel shaft of the applicator that needs to be inserted into the soil. For application of EDB the steel shaft was pushed vertically into the soil up to the restraining ring (adjusted to an application depth of 30 cm). The injector handle was then pushed to release the required dosage rate through the release holes that are situated at the tip of the shaft. Applications were done in parallel rows. Injections within each row were 30 cm apart and rows were spaced 30 cm apart. Escape of gas from the soil was minimised by stepping onto the injection hole with a rubber-soled shoe immediately after release of the product into the soil. A second tarpaulin was used to cover the freshly fumigated soil to further prevent gas from escaping and to prevent contamination of the treated soil. Three weeks after fumigation the soil was used to fill microplots or pots for greenhouse trials.

To minimise the risk of EDB residual effects (Nel *et al.*, 2007) soil in the microplots was re-fumigated (Fig. 2.4) three weeks before planting of each trial. The fumigation procedures followed in microplots were similar to those of the soil spread out on a tarpaulin described above.

Figure 2.4. Operators wearing full protective clothing during fumigation of soil with EDB in the microplots.

2.2.2 SOIL NUTRIENTS

Before each trial was planted a soil nutrient analysis was done by the Soil Science Laboratory of the ARC-GCI, Potchefstroom. Nutrients were added based on a soil analysis and nutrient guidelines for groundnut production (Swanevelder, 1997). Nutrient requirements for each trial will be provided in the respective chapters. Nutrients were incorporated into the soil contained in pots (greenhouse) or microplots as follows:

2.2.2.1 Greenhouse

The soil in each pot was emptied and spread onto a thoroughly cleaned plastic tray. The required nutrients were sprinkled over the soil surface and thoroughly mixed with the soil by hand. The pot was then refilled with the soil and nutrient mixture. After each mixing the trays and gloved hands of the operator were cleaned.

2.2.2.2 Microplots

Nutrients were incorporated into the soil in the microplots as demonstrated in Figure 2.5. First the required amount of nutrients was evenly broadcast over the soil surface and then worked into the top 30 cm of the soil with a garden fork.

Figure 2.5. Nutrients are incorporated into the top 30 cm of the soil with a garden fork.

2.2.2.3 Field sites

Methods and rates of nutrients applied at the research station at Jan Kempdorp (27.95°S, 24.85°E) and on a producer's farm near Hartswater (27.83°S, 24.79°E) were based on those used for the respective localities and as recommended after soil analyses. The soil type at both localities is Hutton. Herbicides were applied by a tractor-mounted implement as and when required (Fig. 2.6).

Figure 2.6. Application of herbicides with a tractor-mounted implement.

2.3 BIOLOGICAL MATERIALS

2.3.1 GERMPLASM

2.3.1.1 Seed

The germplasm bank of the Groundnut Breeding Unit of the ARC-GCI provided seed of groundnut genotypes used in the first microplot trial during the evaluation of *D. africanus*. Seed of genotypes in the second microplot trial that replaced genotypes that did not show *D. africanus* resistance in the first microplot trial (Chapter 3), were also obtained from the Groundnut Breeding Unit. However, seed required of the breeding lines PC254K1 and PC287K5 and the cultivars Sellie and Kwarts in the second microplot trial (Chapter 3) and for the rest of the greenhouse, microplot and

field trials during this study were produced in the uninoculated section (nematode-free section) of the first microplot trial (Chapter 3).

2.3.1.2 Seed treatment

Pots used in greenhouse trials and the plots in microplot trials were watered a day before planting to enhance germination of the seed (Swanevelder, 1997). Standard treatments used in groundnut production were adopted for all seed used in the trials during this study. The recommended dosage rate of the standard fungicide thiolin is based on seed mass (120 g fungicide per 50 kg seed) and it was always applied before planting. For thiolin application the relevant seeds were weighed, placed in a paper bag and the required rate of the powder-formulation fungicide was added. To ensure proper coating of the seeds by the fungicide, each paper bag containing seeds and fungicide was shaken rigorously for at least 30 s. Protective gloves and face masks were used throughout the process to protect the operator from contact by and inhalation of the fungicide. Treated seeds were planted directly afterwards by hand to the depth of 5 cm in soil and inter- and intra-row spacing relevant to the specific method of groundnut production (Swanevelder, 1997).

The required amount of *Bradyrhizobium arachis* nitrogen-fixing bacteria were added onto the treated seed before nematode inoculation. The recommended dosage rate for the nitrogen-fixing bacteria is 250 g per 50 kg seed.

2.3.2 NEMATODES

Microplot, greenhouse and growth cabinet trials were inoculated with nematodes extracted from *in vitro D. africanus*-groundnut-callus-tissue cultures (Fig. 2.7 A) that are maintained in a growth cabinet (Fig. 2.7 B) at a constant temperature of 26 °C (Van der Walt & De Waele, 1989).

Figure 2.7. A. *Ditylenchus africanus* groundnut callus tissue cultures such as those used for the inoculation of microplot and greenhouse trials in this study.
B. Growth cabinets used for the incubation of *Ditylenchus africanus* groundnut callus tissue cultures.

2.3.2.1 Nutrient medium and callus tissue culturing

The nutrient medium for groundnut callus tissue culturing (Van der Walt & De Waele, 1989) used during this study was prepared as follows: 4.4 g basal salt mixture, 1 ml vitamin B5 complex, 30 g sucrose and 0.9 g caseinhydrolysate were transferred to a 1-liter glass flask. The flask containing the latter ingredients was topped up with distilled water to 1 l. The contents of the flask were mixed thoroughly with a magnetic stirrer. During the mixing procedures the pH of the mixture was adjusted to 5.8 using 1 molar HCl / NaOH. Gelrite (8 g) was added to the mixture after having adjusted its pH. The mixture was then autoclaved at 120 °C for 15 min.

From here on were conducted under a laminar flow cabinet in sterile conditions. The autoclaved mixture was left to cool down for approximately 10 min before injecting 5 ml kinetin and 10 ml 2.4-D through millipore filters into the cooled mixture. The latter two ingredients completed the medium and were thoroughly mixed with the contents by gently shaking of the flask by hand. The medium was immediately poured into 65-mm petri dishes, each dish receiving approximately 20 ml medium. The medium contained in each petri dish was allowed to cool in the laminar flow cabinet for $\pm$ 20 min before used for transferring of the callus tissue.

Callus was propagated on the growth medium described above from young, surface-sterilised groundnut leaves (Van der Walt & De Waele, 1989) collected from the cultivar Sellie planted in a greenhouse for this purpose. Surface sterilisation of the

leaves proceeded as follows: freshly collected leaves were rinsed under a gentle stream of tap water to remove any unwanted materials from their surfaces and then transferred to a glass beaker that contained a 70 % ethanol solution. The leaves were allowed to soak in the ethanol solution for 30 s. After 30 s the ethanol solution was carefully poured off until only the leaves remained in the beaker. A 2-% NaOCl solution mixed with two drops of dishwashing liquid was then poured into the beaker onto the leaves, which were allowed to soak in the latter solution for 15 min. The NaOCl solution was then carefully poured off from the leaves and replaced with sterilised, distilled water. The latter rinsing process was repeated four times with sterilised distilled water to remove all remaining traces of the NaOCl solution, which completed the surface-sterilisation process of the leaves. The sterilised leaves were cut on a sterilised glass pane with a sterilised scalpel into 1-cm^3 pieces. Each piece was transferred from the glass pane with a sterilised pincet onto a growth medium contained in a petri dish. The petri dish was then sealed with parafilm. Growth of the callus was promoted in darkness for four months in a growth cabinet (Fig. 2.7 B) at a constant temperature of approximately 26 °C.

2.3.2.2 Inoculation of groundnut callus tissue with *D. africanus*

All the *D. africanus* groundnut callus tissue cultures were initiated using nematodes extracted from pods (Bolton *et al.*, 1990) collected from *D. africanus*-infested fields. Extracted nematodes were surface-sterilised (Meyer, 1984) before being inoculated on the callus tissue. Each nematode was picked with a sterilised needle from a petri dish containing soaked pod tissue and transferred to a 5-ml centrifugal tube that contained 2 % streptomycin sulphate. The nematodes were allowed to settle at the bottom of the centrifugal tube in the streptomycin sulphate solution for 24 h. After 24 h the streptomycin sulphate was carefully pippetted off using a fine sterilised pasteur pipette and replaced with sterilised, distilled water pipetted onto the nematodes in the centrifugal tube. The nematodes were then allowed to re-settle at the bottom of the centrifugal tube before repeating this process two more times with distilled water to remove all remaining traces of the streptomycin-sulphate solution.

2.3.2.3 Renewal of *D. africanus* callus tissue cultures

Maintenance of *D. africanus* callus tissue cultures requires renewal of each culture every three to four months. Mature *D. africanus* cultures are presented in Fig. 2.8 A & B. Renewal of *D. africanus* callus tissue cultures was done as described by Van der

Walt and De Waele (1989). Callus and nutrient medium (Fig. 2.8 B) were cut into 10- to 20-mm pieces, which contained *D. africanus* eggs, juveniles and adults. All pieces of callus tissue and medium were used to inoculate a fresh callus growing on a growth medium (Fig. 2.8 A). Petri dishes containing freshly inoculated callus were sealed with parafilm and incubated in darkness at 26 °C in a growth cabinet.

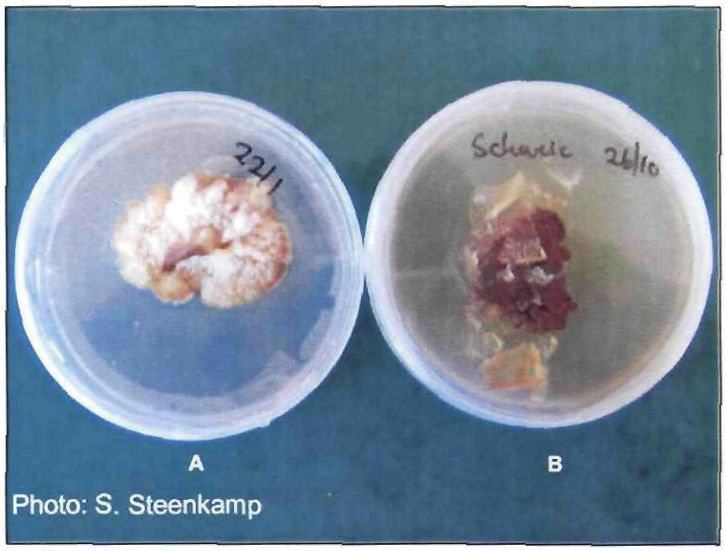

Figure 2.8. A. A mature four-month-old groundnut callus.
B. A *Ditylenchus africanus*-infested groundnut callus tissue culture due for renewal.

2.3.2.4 Extraction of *D. africanus* from callus tissue

Nematodes were recovered from the cultures according to the procedures of De Waele and Wilken (1990). Nematodes present on the lid of the petri dish and on the surface of the intact callus tissue and growth medium were rinsed with a slow trickle of ± 20 ml tap water into a calibrated 500-ml glass beaker. The callus tissue together with growth-medium pieces was then sectioned with a scalpel, placed on a 710-µm pore sieve nested on a 45-µm-mesh sieve and rinsed with tap water. This allowed the cells of the callus tissue to separate so that the nematodes within the tissue and in the growth medium could be rinsed through the 710-µm-mesh sieve and collected on the 45-µm-mesh sieve. These nematodes were washed into the 500-ml glass beaker that already contained the nematodes from the petri-dish lid and the surface of the callus tissue and growth medium.

2.3.2.5 Determination of the number of nematodes extracted from callus tissue

The 500-ml beaker containing the extracted nematodes described above was topped up with tap water to 300 ml. Nematodes were kept suspended in the water by means of a magnetic stirrer to ensure that sub-samples were representative of the nematode population in the suspension. Three 10-ml sub-samples of the nematode suspension were transferred with a pipette from the beaker into counting dishes while stirring the suspension. Eggs, juveniles and adults in each 10-ml sub-sample were counted using a research microscope and the number of nematodes present in each counting dish was used to calculate the mean number of nematodes per 10 ml nematode suspension: (number of nematodes in sub-sample 1 + number of nematodes in sub-sample 2 + number of nematodes in sub-sample 3) / 3. This figure was used to calculate the total number of nematodes present in the 300-ml nematode suspension according to the equation y = n x 300 ml / 10 ml where: y = number of nematodes per 300 ml and n = mean number of nematodes per 10-ml nematode suspension.

2.3.2.6 Preparation of nematode inoculum and inoculation procedures

The desired number of nematodes to be inoculated per plant / plot was always suspended in 20-ml aliquots of water. To achieve this the 300-ml nematode suspension describe above was diluted to a volume that would yield the desired number of nematodes needed to inoculate each plant / plot suspended in 20 ml water. When insufficient numbers of nematodes were present in the 300-ml suspension another callus culture was put through the extraction process as described under 2.3.2.4. Plants were inoculated at planting by pipetting the required volume of nematode-suspension evenly over the seeds.

2.4 DATA COLLECTION AND TRIAL ASSESSMENT PROCEDURES

2.4.1 Sampling

2.4.1.1 Greenhouse trials

The aboveground parts of plants were cut off and discarded. Thereafter every pot was emptied separately onto a clean plastic tray so that the whole pod system of each plant could be collected from the soil. The pod system was placed in a marked

plastic bag after removal of the roots from the system. Soil and roots were then discarded. Pod samples were kept moisture-free and stored at 4 °C until nematode extraction. Nematodes were extracted from the pod tissues within five days of sampling.

2.4.1.2 Microplot and field trials

For microplot and field trials plants were carefully lifted with a garden fork at harvesting, taking care to prevent pods from breaking off in the soil. Aboveground parts of randomly selected plants collected for nematode assessments were removed and discarded. In the microplot trials whole pod systems of all plants were collected and stored as described above. Roots of plants from the field trails were left attached to the pod system and together with a 200-ml sub-sample of soil collected from the root zone of the plant, placed into a marked plastic bag because nematode assessments were also made in root and soil samples to determine nematode species other than *D. africanus* present in the field. Samples were stored at 4 °C until extraction, which was within three days of sampling.

Plants used for yield assessment in microplot and field trials were lifted from the soil as described above. The pods were removed and transferred to appropriately marked paper bags. The rest of the plants were discarded. The bags were placed in a greenhouse for approximately seven days to allow air-drying of the pods at an ambient temperature of between 18 and 27 °C.

2.4.2 Nematode extractions

2.4.2.1 Soil samples

Nematodes were extracted from soil samples according to the sugar-flotation method described by Jenkins (1964). Soil samples were sub-sampled to 200 cm^3 and thoroughly mixed with 400 ml tap water contained in a 5-l, calibrated beaker. The suspension was poured through a course kitchen sieve into another 5-l container to get rid of grit and debris. Then it was stirred and allowed to settle for 30 s. The supernatant was then poured through a 750-µm-mesh sieve nested on a 45-µm-mesh sieve. The residue that remained behind on the 45-µm-mesh sieve was poured into a 50-ml centrifuge tube and was centrifuged. The supernatant was discarded

and replaced by a 1.13 g / cm^3 sucrose solution (624 g sucrose dissolved in 1 l tap water), which was thoroughly mixed with the sediment in each tube and centrifuged for 1 min at 2 500 revolutions per minute. The supernatant was poured onto a 45-µm-mesh sieve and residual sucrose rinsed from the nematodes with a gentle stream of tap water. Nematodes were then washed into a 130-ml plastic sample flask with ± 20 ml tap water and stored at 4 °C. Nematodes in each sample were counted using a research microscope within three days of extraction. Plant-parasitic nematodes other than *D. africanus* collected from the soil samples of the field trials were identified to genus level and expressed as nematodes per 200 cm^3 soil.

2.4.2.2 Root and peg samples

The method used for the extraction of *D. africanus* from roots and pegs was described by De Waele *et al.* (1987). All root and peg tissues were removed from plants sampled from microplot and field trials and cut into 1-cm pieces. These pieces were thoroughly mixed by hand and sub-sampled to 5 g. The sub-samples were each macerated for 2 min in 250 ml tap water using a high-speed, domestic blender. The macerated tissue was poured onto a 750-µm-mesh sieve nested on a 45-µm-mesh sieve. Course plant material that collected on the 750-µm-mesh sieve was discarded after it was thoroughly rinsed with tap water and the residue from the 45-µm-mesh sieve was washed into a 50-ml centrifugal tube. Four centrifugal tubes containing the residue from the 45-µm-mesh sieve of four 5-g root or peg samples were topped up to 50 ml with tap water. Kaolin (2 cm^3) was added and thoroughly mixed with the water and residue. The four mixtures were centrifuged for 5 min at 2 000 revolutions per minute. During centrifugation the kaolin trapped the nematodes at the bottom of the tube. The supernatant in each centrifuge tube was discarded.

Nematodes trapped at the bottom of each tube were separated from the kaolin using the sugar-flotation method described previously. Nematodes were washed into a 130-ml plastic sample flask in ± 20 ml tap water. After storage at 4 °C in a fridge the water containing the nematodes was poured into a counting dish and specimens were counted using a research microscope within three days of extraction. Eggs were not included since the developing juveniles in the eggs could not be identified. Nematodes were expressed as number per 5 g roots or 5 g pegs.

2.4.2.3 Hull and kernel samples

Pods of plants collected for nematode assessments were removed by hand from lifted plants. The pods were mixed and 20 were randomly picked. Hull and kernel tissue was extracted separately according to the procedures described by Bolton *et al.* (1990). Hulls were broken by hand and kernels were cut with a scalpel into small pieces to facilitate migration of the nematodes from the plant tissue into the water. The tissue was soaked for 24 h in petri dishes containing ± 20 ml tap water. After 24 h the contents of each petri dish were poured onto a 750-µm-mesh sieve nested on a 45-µm-mesh sieve. Course plant material collected on the 750-µm-mesh sieve was discarded after thoroughly washing it through with tap water. The nematodes collected during this process on the 45-µm-mesh sieve were washed into a 130-ml plastic sample bottle with ± 20 ml water. After storage at 4 °C the contents of each sample bottle were poured into a counting dish and the nematodes counted using a research microscope within three days after extraction. Nematode numbers were expressed per 5 g hulls or 5 g kernels. Numbers of *D. africanus* present per 15 g pods (Pf) was calculated by adding the number of *D. africanus* per 5 g pegs, 5 g kernels and 5 g hulls.

2.4.3 Crop yield

2.4.3.1 Yield quantity

To determine yield mass per replicate the procedures described by Mc Donald (1998) were followed. Pods collected from plants sampled for yield assessments were weighed and the pod mass for each replicate was recorded. A sub-sample of 500 g pods per replicate was shelled and the kernels were used to determine yield quality.

2.4.3.2 Yield quality

The quality of each kernel-yield replicate was determined in a 200-g kernel sub-sample obtained from the shelled 500-g pod samples described above. These sub-samples were subjected to grading procedures stipulated by the Act on Agricultural Product Standards, 119 of 1990 (SA, 1997). Each 200-g sub-sample was placed on sieves nested on each other in receding order of aperture size. A solid pan was

placed underneath the 7.20-mm-aperture slotted sieve. Kernels were sifted through 6.75-mm, 6.00-mm and 7.20-mm-aperture slotted sieves, similar to those used in the groundnut industry. The kernels remaining on the 6.75-mm-aperture and 6.00-mm-aperture sieves were sorted into unsound, blemished, soiled, damaged, shrivelled and sun-dried categories. Kernels on the 7.20-mm-aperture sieve were sorted into unsound, blemished and unsplit. Standards used for the grading of kernels are specified as follows:

- *Blemished kernels*: Whole kernels with coloured blotches or streaks that are not associated with the specific groundnut genotype.
- *Damaged kernels*: Broken or whole kernels of which the testa are loose, cracked or have partially or entirely come off.
- *Mould-infested kernels*: Those kernels on which external or internal mould growth is visible with the naked eye or those kernels damaged or discoloured by mould growth.
- *Shrivelled kernels*: Whole kernels with a shrivelled, grooved or dented appearance.
- *Soiled kernels*: Whole kernels that are soiled to such an extend that their appearance is affected.
- *Sun-dried kernels*: Those kernels of which the testa comes off easily when lightly rubbed by hand.
- *Unsound kernels*: Mould-infested kernels or kernels that are decayed, chalky, have sprouted or are discoloured and are not characteristic of healthy kernels of the specific genotype.

The different grades of kernels from each sieve were weighed separately and evaluated as stipulated by the abovementioned products standard act (Table 2.1).

Table 2.1. Local specifications for groundnut grading as stipulated by the Act on Agricultural Products Standards, 119 of 1990 (SA, 1997).

Grade	Sieve slot (mm)	Seed on sieve (%)	UBS (%)	Unsound (%)	Total defects (%)
Choice	6.75	> 20	< 10	$\leq$ 5	< 20
Standard	6.75	> 10	< 20	$\leq$ 10	< 35
Diverse	6.00	> 40	< 30	< 15	< 35
Crushing	7.20	>20	> 30	> 15	> 35

2.5 STATISTICAL ANALYSES

Degrees of freedom (D.F.) (error) $\geq$ 18 (Van Ark, 1981) were pursued in all trials. Data were entered on a spreadsheet of Microsoft Excell before being imported into Stat Graphics 5 Plus for Windows. The appropriate analysis of the nematode data will be discussed in each relevant chapter. Nematode data were ln(x+1) transformed before statistical analysis unless stated otherwise. Final nematode number (Pf) per 15 g pods was used as indicators of resistance or susceptibility of each groundnut genotype because *D. africanus* populations peak at harvest time in pods (Basson *et al.*, 1990). Unless otherwise stated nematode numbers from root and soil samples were not determined because *D. africanus* reproduces poorly in roots (Van der Walt & De Waele, 1989; Basson *et al.*, 1990 & 1991; De Waele *et al.*, 1990; Jones & De Waele, 1990) and numbers in the soil are small and variable (Venter *et al.*, 1992). The reproduction factor (RF) of *D. africanus* on each groundnut genotype was determined in all the trials using Oosterbrink's equation: RF = final population density (Pf) / initial population density (Pi) (Windham & Williams, 1987). The RF of *D. africanus* on groundnut genotypes was determined in most trials because it provides a basic measurement for the reproduction of a nematode population on a crop (Windham & Williams, 1987; Marin *et al.*, 1999) and supports Pf data. The Pi and Pf used in the equations were not ln(x+1) transformed because measurements (Pi and Pf) of a variable (RF) must be in a nominal scale (Van Ark, 1981).

Yield mass, yield quality and income for microplot trials as specified in the relevant chapters were calculated using Maksi Plan, which is a computer programme developed by the ARC-GCI for the evaluation of crop cultivars and provides functions and evaluations specifically applicable to this study. Calculations of Maksi Plan were based on yield quantity and quality data determined for each genotype and the grades were priced according to the local market value for groundnut at the time of the study.

CHAPTER 3

IDENTIFICATION OF GROUNDNUT GENOTYPES WITH RESISTANCE TO *DITYLENCHUS AFRICANUS*

3.1 INTRODUCTION

Currently available control measures are not adequate under all conditions to keep *D. africanus* numbers below damage-threshold levels (Basson *et al.*, 1990 & 1993; Mc Donald *et al.*, 2005). This nematode is difficult to control because of its ability to survive in the absence of groundnut (Basson *et al.*, 1990; De Waele *et al.*, 1990 & 1991; Swart & Jones, 1994), its high reproductive potential and its short life cycle (De Waele & Wilken, 1990). These characteristics enable the nematode to quickly build up to large populations that cause severe damage (De Waele & Wilken, 1990; De Waele *et al.*, 1990; Venter *et al.*, 1991; Basson *et al.*, 1990; 1992 & 1993, Mc Donald *et al.*, 2005).

Cultivation of resistant crops or cultivars is one of the easiest ways to achieve a reduction in initial nematode densities (Brown, 1987) and it provides an effective alternative for the management of various plant-parasitic nematodes. Resistant plant material is able to resist ingress, establishment and spread of the parasite itself (Bos & Parlevliet, 1995) and keep the latter below damage-threshold levels (Roberts & May, 1986; Fourie *et al.*, 1999; Ogallo *et al.*, 1999; Timper *et al.*, 2003; Todd *et al.*, 2003; Fourie, 2005). The same principle should be applicable to *D. africanus* management on groundnut (De Waele *et al.*, 1990).

For the successful implementation of IPM programmes, however, it is also important to obtain information on the minimum nematode population densities that cause measurable damage (damage-threshold levels) on a crop (Barker & Nusbaum, 1971). If a nematode population could be kept below this damage-threshold level, the particular control measure is considered successful (Kim & Ferris, 2002). Damage-threshold levels of several plant-parasitic nematode species have been studied on a wide variety of crops (Ferris, 1978 & 1985; Niblack *et al.*, 1986b; Koenning, 2000; Kim & Ferris, 2002; Pérez *et al.*, 2003; Fourie, 2005; Mc Donald *et al.*, 2005). Greenhouse studies showed that damage by *D. africanus* on groundnut could become significant at an initial population density (Pi) of 50 nematodes (Venter *et al.*,

1991), while microplot studies indicated that a Pi of 250 to 750 nematodes will lead to downgrading of the cultivar Sellie to standard grade (Mc Donald *et al.*, 2005).

Currently there are no cultivars available that have resistance to *D. africanus*. Previously more than 600 genotypes were evaluated for resistance to this nematode but none proved useful (Basson *et al.*, 1991; Van der Merwe & Joubert, 1992). Consequently, no information is available in the literature on the comparative effect of increments of Pi of *D. africanus* on susceptible, tolerant or resistant groundnut genotypes. The ultimate challenge for any breeding-programme is, furthermore, to release new cultivars through the shortest route possible. One possible approach could be to reduce the period of microplot and field evaluations by using callus tissue to screen genotypes against *D. africanus*. The objectives of this part of the study were to i) identify groundnut genotypes with resistance to *D. africanus* that would also be sustainable under field conditions, ii) compare the reproduction and damage-threshold levels of *D. africanus* on resistant, susceptible and tolerant groundnut genotypes in microplots and iii) determine whether resistance expressed in pods of a resistant genotype is transferred to its callus tissue under controlled conditions.

3.2 MATERIALS AND METHODS

3.2.1 Identification of *D. africanus*-resistant groundnut genotypes

To achieve the first objective selected groundnut genotypes were evaluated over two consecutive seasons in microplot and field trials. The genotypes selected for evaluation for *D. africanus* resistance are listed in Table 3.1. The genotypes were selected on the basis of characteristics such as high oleic-acid contents or disease resistance (Cilliers *et al.*, 2001). Kernels of genotypes with a high oleic-acid content are less reactive with oxygen and consequently have a longer shelf life (Isleib *et al.*, 1994), while the use of genotypes that have resistance to one or more diseases can, potentially, reduce input costs for producers (Dickson & De Waele, 2005).

Table 3.1. Origin and preferred characteristics of groundnut genotypes that were selected for the identification of resistance to *Ditylenchus africanus*.

Genotype	Origin	Source	Preferred characteristic
*Sellie	Locally bred	ARC-GCI[1]	*D. africanus*-susceptible standard
*Kwarts	Locally bred	ARC-GCI	*D. africanus*-tolerant standard
*Harts	Locally bred	ARC-GCI	resistant to black pod rot
*UF85	USA	Unknown	high oleic acid content
*JL24	Congo	SARCCUS[2]	resistant to tomato spotted wilt virus
*CG7	Malwi	ICRISAT[3]	resistant to a variety of diseases
**453	Senegal	Unknown	high oleic-acid content
**73-30	Senegal	Unknown	high oleic-acid content
**PC223	Locally bred	ARC-GCI	high oleic-acid content
**PC299K5	Locally bred	ARC-GCI	high oleic-acid content
**PC254K1	Locally bred	ARC-GCI	high oleic-acid content
**PC287K5	Locally bred	ARC-GCI	suspected resistance to *D. africanus*

*Cultivar

**Breeding line

[1]Agricultural Research Council-Grain Crops Institute, Potchefstroom, North West Province

[2]Southern African Regional Commission for the Conservation and Utilisation of Soil

[3]International Crops Research Institute for the Semi-Arid Tropics

3.2.1.1 Trial layout

3.2.1.1.1 Seed and genotypes

Seed of the genotypes that were tested in this study were obtained from the germplasm bank of the groundnut-breeding department or from nematode-free nurseries (Chapter 2). Before planting 5-g sub-samples of the seed of each genotype were soaked in water for 24 h (Bolton *et al.*, 1990) to ascertain that the seed was *D. africanus*-free. The rest of the seed was treated with fungicide and inoculated with *Bradyrhizobium arachis* nitrogen-fixing bacteria (Chapter 2) before planting.

The groundnut genotypes tested in each microplot or field trials during each season are listed in Table 3.2. The cultivar Sellie served as *D. africanus*-susceptible standard (Mc Donald, 1998) in all trials. Kwarts, classified as tolerant to *D. africanus* (Mc

Donald, 1998) was used as the other standard because no source of resistance to *D. africanus* was available at the onset of this study.

Table 3.2. Groundnut entries for the identification of resistance to *Ditylenchus africanus* in the respective microplot and field trials conducted over two consecutive growing seasons (2003-2004 – 2004-2005).

Microplot trial 1 (2003-2004)	Microplot trial 2 (2004-2005)	Field trials (2) (2004-2005)
Sellie [1]	Sellie	Sellie
Kwarts [2]	Kwarts	Kwarts
UF85	JL24	JL24
73-30	Harts	Harts
453	CG7	CG7
PC223	PC299K5	PC299K5
PC254K1	PC254K1	PC254K1
PC287K5	PC287K5	PC287K5

[1] *D. africanus*-susceptible standard

[2] *D. africanus*-tolerant standard

3.2.1.1.2 Microplot trials

The two microplot trials were planted in a facility that consists of a row of 20 rectangular clay-brick troughs (Chapter 2). The 20 troughs were each filled with fumigated, sandy-loam soil (Chapter 2). The soil was fumigated once more in the microplots three weeks before planting with EDB as described in Chapter 2. Nutrients were added to each trough according to the procedures described in Chapter 2. Requirements for the 2003-2004 microplots were 150 g dolomitic lime, 130 g super phosphate (10.5 % P), 40 g sodium chloride (KCl) and 35 g calcium nitrite ($CaNO_3$) per trough. Additional nutrients required for the microplot trial during 2004-2005 were 200 g dolomitic lime, 139 g super phosphate (10.5 % P), 42 g KCl and 40 g $CaNO_3$ per trough.

The trials were planted in a randomised complete split-plot design. Factor 1 consisted of 10 plots artificially inoculated with *D. africanus* and 10 uninoculated plots. The inoculated section received $\pm$ 3 000 *D. africanus* of various life stages per plant at planting. The nematodes used for inoculation were extracted from *in vitro D.*

africanus cultures prepared for inoculation according to procedures described in Chapter 2. Factor 2 consisted of the relevant eight genotypes evaluated during each season (Table 3.2). Each genotype, inoculated and uninoculated was replicated five times.

The trial layouts were the same in 2003-2004 and 2004-2005, although the randomisation in each block was different in the two seasons. Four rows were planted in each trough with an inter-row spacing of 45 cm (Chapter 2). Each plant row contained 20 seeds of each respective genotype. Seed was planted to a depth of 5 cm using an intra-row spacing of 5 cm (Chapter 2).

3.2.1.1.3 Field trials

Sustainability of resistance of these genotypes under field conditions was tested in two separate field trials. Fields naturally infested with plant-parasitic nematodes were selected and were used as sites for the trials. One field trial was planted on an overhead-irrigated site near Hartswater (27.83°S, 24.79°E) and the other trial on a flood-irrigated site located on the ARC Research Station (Fig 3.1) near Jan Kempdorp (27.95°S, 24.85°E).

Figure 3.1. A groundnut field trial at Jan Kempdorp.

No *D. africanus* was artificially added to the soil since both sites were previously planted with groundnut and were naturally infested with this nematode. Soil at these sites was not treated with any nematicide or fumigant at or after planting of the trials

because of the risk of contamination of adjacent plots by chemical treatments, particularly under irrigation, which could interfere with results (Hough & Thomason, 1975). Nematode-free controls were, therefore, not included in the field trials.

The trials were planted in a randomised complete block design (RCBD) including eight treatments (genotypes listed in Table 3.2) and six replicates per treatment. Each replicate consisted of eight 1-m rows planted at an inter-row spacing of 45 cm (Chapter 2). Each row was planted to 20 seeds of each respective genotype. The seeds were planted to a depth of 5 cm with intra-row spacing of 5 cm (Chapter 2).

3.2.1.2 Collection of nematode and yield data

Nematode and yield assessments were done at harvesting. Four randomly selected plants from each row were collected for nematode extraction from the inoculated section of the microplot and from all plots in the field trails. Extractions were also made from four randomly selected plants from the uninoculated section of the microplot trials to confirm that this section remained nematode-free.

Separate extractions were done from peg, hull and kernel samples from the microplot trials and from soil, root, peg, hull and kernel samples from the field trials, following the procedures described in Chapter 2. Nematodes were counted using a research microscope and the numbers (Pf) were added and expressed as number of *D. africanus* per 15 g pods (Chapter 2).

In the field trials root and soil samples were included because assessments were made of all the nematode species that occurred along with *D. africanus*. Nematodes were extracted from soil and plant tissues according to the methods described in Chapter 2. Two different methods were used for extraction of nematodes from root samples collected from the field trials. The maceration method (De Waele *et al.*, 1987) was used to extract plant-parasitic nematodes other than *Meloidogyne* spp. from root samples, which were expressed as nematodes per 5 g roots (Chapter 2). However, the latter method is not effective for extracting eggs and second-stage juveniles (J2) of *Meloidogyne* spp. from root samples. An additional NaOCl method (Riekert, 1995) was used for the extraction of root-knot nematode eggs and J2 from root samples, which were expressed as *Meloidogyne* spp. per 50 g roots. The NaOCl method provided a more reliable estimate of the *Meloidogyne* spp. population

densities present in the field. All plant-parasitic nematodes other than *D. africanus* were identified to genus level.

The 16 remaining adjacent plants in each row that were not used for nematode extractions were collected for yield assessment in the microplot as well as field trials. Yield quality (microplot and field trials) and quantity (microplot trials) assessments were made as described in Chapter 2. Yield of the genotypes in the uninoculated section of the microplot trials served as nematode-free control to distinguish between resistant and tolerant plant responses.

3.2.2 Reproduction and damage-threshold levels of *D. africanus* on resistant, susceptible and tolerant groundnut genotypes

The microplot facility used for the second part of the study during 2004-2005 consists of concrete pipes arranged vertically in 15 rows of eight pipes each (Chapter 2). The 120 plots were each filled with the same fumigated, sandy-loam soil (Chapter 2) that was used to fill the microplots in the evaluation of the groundnut genotypes against *D. africanus*. Procedures for the fumigation of the soil with EDB are described in Chapter 2. The nutrients incorporated into the soil of each plot (Chapter 2) at the beginning of the 2004-2005 growing season were 78 g dolomitic lime, 47 g superphosphate (10.5 % P), 31 g KCl and 20 g $CaNO_3$.

To accommodate the total number of treatments (192) in the 120 available plots, a 0.4 x 1 m^2 piece of rigid, 1.5-cm thick rubber mat was installed vertically along the diameter of each plot after application of nutrients to the soil. The rubber mats divided each pot in equal halves and served as a physical barrier for water and nematodes between the two halves. Each plot halve therefore served as a separate plot. Each plot was planted with six seeds of a groundnut genotype. The seed was planted to a depth of 5 cm (Chapter 2).

Seeds of Sellie, Kwarts, PC254K1 and PC287K5 were obtained from nematode-free seed nurseries (Chapter 2). Before planting 5-g sub-samples of the seed of each of the groundnut genotypes were soaked in water for 24 h (Bolton *et al.*, 1990) to ascertain that the seed was free of *D. africanus* infection. The rest of the seed was then treated with fungicide and *B. arachis* as described in Chapter 2.

The trial was laid out in a RCBD. The four groundnut genotypes were each inoculated (Chapter 2) with 0, 50, 250, 1 000, 2 500, 5 000, 10 000 or 20 000 nematodes (Pi) that originated from Hartswater per plant at planting. Each Pi for each genotype was replicated six times. A plastic marker placed in each plot indicated the respective genotype, the Pi and replicate number. The trial received supplementary irrigation as described in Chapter 2.

Nematode and yield assessments were made at harvesting. Two plants per plot were randomly picked for extraction of nematodes from peg, hull and kernel samples (Chapter 2). The nematodes were counted using a research microscope and expressed as *D. africanus* per 15 g pods (Pf) (Chapter 2). The four plants remaining per replicate were used for yield quantity and quality assessments, following the procedures described in Chapter 2.

3.2.3 Expression of *D. africanus* resistance in callus tissue

To achieve the third objective of this study the reproduction of *D. africanus* was studied on callus tissue from Sellie, Kwarts, PC254K1 and PC287K5 in a growth cabinet trial during September and October 2008. The trial was laid out in a RCBD with the four different genotypes as treatments, each replicated six times. The callus tissue was initiated from young groundnut leaves from each genotype as described in Chapter 2. Each six-week old callus was inoculated with 10 adult *D. africanus*, consisting of five males and five females extracted from an *in vitro D. africanus* culture that originated from Hartswater (Chapter 2). Each petri-dish containing the inoculated callus was sealed with parafilm. Dishes were marked appropriately to indicate the relevant genotype and replicate number. The cultures were incubated in darkness for four weeks at 28 °C in a growth cabinet to allow the nematodes to complete a minimum of four life cycles (De Waele & Wilken, 1990). The growth cabinet was set at a constant temperature of 28 °C and was allowed to stabilise for three days before the onset of this trial. After four weeks the nematodes were extracted from each callus according to the method described in Chapter 2. The nematodes were counted using a research microscope and the numbers were expressed as *D. africanus* per 1 g callus tissue.

3.3 STATISTICAL ANALYSES

Unless otherwise stated nematode data were ln(x+1) transformed before being subjected to statistical analysis. Nematode data from the microplot and field trails on *D. africanus* resistance and that of the growth cabinet trial were subjected to an analysis of variance (ANOVA) (Stat Graphics 5 Plus for Windows). Means were separated by an LSD test ($P \leq 0.05$). Nematode data from the microplot trial on the reproduction and damage threshold of *D. africanus* were subjected to linear and non-linear regression analyses (Genstat for Windows).

The following nematode variables were determined: Final population densities (Pf) of *D. africanus* in pods and callus tissue of all trials. The RF of *D. africanus* on each groundnut genotype was determined for the microplot and growth cabinet trials as described in Chapter 2. RF values were not determined for field trials since Pi, which is a factor used in the determination of the reproduction factor, cannot be accurately estimated under field conditions (De Waele *et al.*, 1990; Venter *et al.*, 1991; Basson *et al.*, 1992; Taylor *et al.*, 2000).

The percentage reproduction rate (RR) of *D. africanus* populations was determined on pods from each groundnut genotype from the microplot and field trials on *D. africanus* resistance. Although resistance is a relative concept, RR can be used to indicate the level of resistance expressed by the host (Timper *et al.*, 2003) in the absence of an RF value in the field because Pi is not required as a factor to determine RR. The RR of each treatment from the inoculated microplot section as well as the field trials was calculated using the equation RR = Pf (genotype) / Pf (Sellie) x 100. Pi and Pf used in the equation to determine RR were not ln(x+1)-transformed since measurements (Pi and Pf) of the variable (RR) must be in a nominal scale (Van Ark, 1981).

Yield quantity of cultivars and breeding lines were determined to be used in Maksi Plan (Chapter 2) but were not compared with one another in either the microplot or field trials on *D. africanus* resistance or in the microplot trial on reproduction and damage threshold levels because yield is determined by intrinsic agronomic traits (Fourie, 2005). Quantity data of genotypes evaluated for resistance to *D. africanus* in the field were not shown because yield data of the genotypes was not used in any additional calculation to determine the effect of *D. africanus* on their yields. Quantity of the yields produced by the four genotypes at each Pi level in the microplot trial on

reproduction and damage threshold levels was compared within each genotype to determine the effect of this nematode on yield quantity.

Yield quality of each genotype collected from all of the microplot and field trials was determined according to standard grading procedures (Chapter 2). Yield quantity and income produced by each genotype in the inoculated as well as the uninoculated sections of the microplot trials on *D. africanus* resistance and for those at different Pi levels in the microplot trial on reproduction and damage threshold levels were determined by means of Maxi Plan (Chapter 2). For the microplot trials on *D. africanus* resistance yield quantity per hectare for each genotype was calculated according to the equation Py = (Yui-Yi) 100 / Yi, where Py = yield response, Yui = yield in the uninoculated section and Yi = yield in the inoculated section (Weaver *et al.*, 1985). Income difference was calculated using the same equation but replacing yield with income: Pin = (Iui-Ii) 100 / Ii, where Pin = potential income, Iui = income calculated for the uninoculated section and Ii = income calculated for the inoculated section. Loss or gain of yield and income per hectare of each genotype was then compared among the genotypes.

3.4 RESULTS

3.4.1 Identification of *D. africanus*-resistant groundnut genotypes

3.4.1.1 Microplot trials

3.4.1.1.1 Final nematode population densities, reproduction factor and reproduction rate

Genotypes that showed resistance to *D. africanus* during the first microplot trial during 2003-2004 were re-evaluated in the second microplot trial during 2004-2005. Genotypes that did not show any resistance to *D. africanus* in the first microplot trial were replaced by different ones. During both seasons the nematode numbers in the pods of Sellie, Kwarts, PC254K1 and PC287K5 varied in spite of a constant inoculum rate of 3 000 nematodes per plant in the microplot trials. Significantly lower Pf's were extracted from pods of PC254K1 and PC287K5 compared to those of the rest of the genotypes during 2003-2004 (Fig. 3.2).

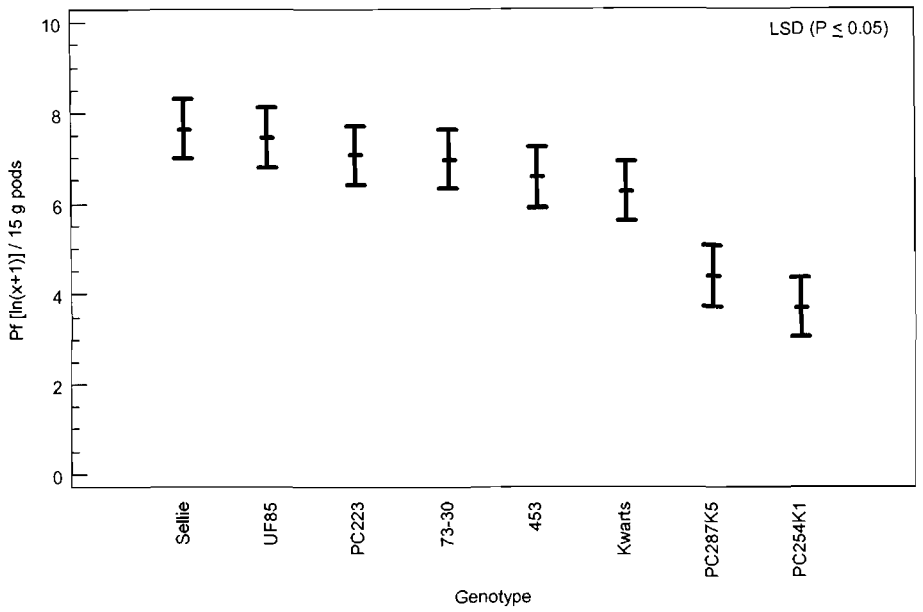

Figure 3.2. *Ditylenchus africanus* numbers (Pf) in pods of eight groundnut genotypes from the inoculated section of a microplot trial during 2003-2004 ($P \leq 0.05$; F-ratio = 10.02).

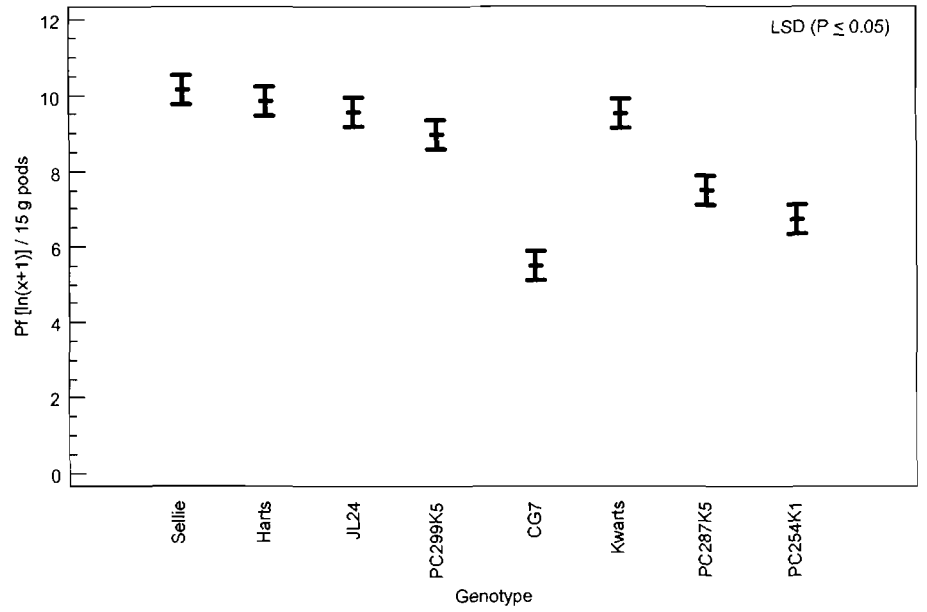

Figure 3.3. *Ditylenchus africanus* numbers (Pf) in pods of eight groundnut genotypes from the inoculated section of a microplot trial during 2004-2005 ($P \leq 0.05$; F-ratio = 40.69).

During 2004-2005 the latter two genotypes along with CG7 maintained significantly lower Pf's compared to those of the rest of the genotypes (Fig. 3.3). The Pf of CG7 was significantly lower than those for the other genotypes tested during 2004-2005.

PC299K5 also had a significantly lower Pf than those of the susceptible checks Sellie and Harts during the same season.

The relatively low Pf present in pods of genotypes in the inoculated section of the microplot trial during 2003-2004 also translated in their respective RF, which were all lower than one (Table 3.3). In spite of the low levels during this season the Pf of PC254K1 was 53.9-fold lower than that of Sellie. During the same growing season the Pf of PC287K5 was 7.2-fold lower than that of Sellie. The RR of PC254K1 and PC287K5 relative to Sellie was also very low. Those of the tolerant Kwarts were less than a quarter of the RR of Sellie, while the RR of the lines 453 and PC233 were less than half that of Sellie.

Table 3.3. *Ditylenchus africanus* numbers (Pf), reproduction factors (RF) and reproduction rates (RR) in pods of eight groundnut genotypes from a microplot trial during 2003-2004.

Genotype	Pf (15 g pods)	RF[1]	RR[2]
UF85	2 700	0.90	100.3
Sellie[3]	2 693	0.89	100
73-30	1 742	0.58	64.7
PC233	1 335	0.45	49.6
453	848	0.28	31.5
Kwarts[4]	610	0.20	22.7
PC287K5	372	0.12	13.8
PC254K1	50	0.02	1.9

[1]RF = Pf / Pi

[2]RR = Pf (genotype) / Pf (Sellie) x 100

[3]*D. africanus*-susceptible standard

[4]*D. africanus*-tolerant standard

During 2004-2005 the Pf's overall were much higher than the previous season in the microplots (Table 3.4). The Pf in pods of CG7 was 103-fold lower, 26.9-fold lower in PC254K1 and 13.4-fold lower in PC287K5 than that in Sellie. The RF's of *D. africanus* were higher than one for the majority of the genotypes. CG7 and PC254K1, PC287K5 had RF < 1. The RR's of *D. africanus* (Table 3.4) for CG7, PC254K1 and PC287K5 were 1 %, 3.7 % and 7.5 %, respectively of that for Sellie. The RR of tolerant Kwarts was only more than half of that of Sellie, despite the much higher Pf in this trial.

Table 3.4. *Ditylenchus africanus* numbers (Pf), reproduction factors (RF) and reproduction rates (RR) in pods of eight groundnut genotypes from a microplot trial during 2004-2005.

Genotype	Pf (15 g pods)	RF[1]	RR[2]
Sellie[3]	28 116	9.37	100
Harts	25 649	8.55	91.2
JL24	15 191	5.06	54.0
Kwarts[4]	14 919	4.97	53.1
PC299K5	7 843	2.61	27.9
PC287K5	2 095	0.70	7.5
PC254K1	1 046	0.35	3.7
CG7	273	0.09	1.0

[1]RF = Pf / Pi

[2]RR = Pf (genotype) / Pf (Sellie) x 100

[3]*D. africanus*-susceptible standard

[4]*D. africanus*-tolerant standard

3.4.1.1.2 Yield assessments

The difference in yield quality in the inoculated and corresponding uninoculated sections of the microplot trials varied between genotypes and over seasons (Tables 3.5 & 3.6). The UBS % of most genotypes in the inoculated section was generally higher than those in the corresponding uninoculated section during both seasons.

During 2003-2004 PC254K1, PC287K5 and Kwarts (tolerant) had UBS % lower than 10 and were comparable between the inoculated and uninoculated sections in this regard (Table 3.5). Greater reduction in UBS % from nematode inoculated to uninoculated were only apparent in PC223, Sellie, UF85 and 73-70. The grades from choice to crushing corresponded with the trend in UBS % (Table 3.5). Choice grade kernels were obtained from the inoculated and the uninoculated sections of PC254K1, PC287K5 and Kwarts. The lines 453 and 73-70 produced standard grade kernels in the inoculated and uninoculated sectiond, while PC233, Sellie and UF85 only yielded standard grade kernels for the uninoculated section. Only diverse and crushing grade were obtained from the inoculated sections of the rest of the genotypes.

Table 3.5. Yield quality of eight groundnut genotypes in a microplot trial during 2003-2004.

Genotype	UBS (%)		Choice (%)		Standard (%)		Diverse (%)		Crushing (%)	
	I[1]	UI[2]	I	UI	I	UI	I	UI	I	UI
PC223	25	12	0	0	0	66	73	18	27	16
Sellie[3]	23	14	0	0	0	68	79	21	21	11
UF85	22	2	0	0	0	14	76	21	24	65
73-70	21	12	0	0	46	61	22	21	32	18
453	15	14	0	0	47	56	18	23	35	21
Kwarts[4]	6	5	75	80	0	0	12	9	13	11
PC287K5	1	4	48	31	0	0	36	33	16	36
PC254K1	0	0	64	64	0	0	15	9	21	27

[1]Inoculated

[2]Uninoculated

[3] *D. africanus*-susceptible standard

[4]*D. africanus*-tolerant standard

Table 3.6. Yield quality of eight groundnut genotypes in a microplot trial during 2004-2005.

Genotype	UBS (%)		Choice (%)		Standard (%)		Diverse (%)		Crushing (%)	
	I[1]	UI[2]	I	UI	I	UI	I	UI	I	UI
Harts	26	20	0	0	0	0	66	68	34	32
PC299K5	21	20	0	0	0	0	52	49	48	51
Sellie[3]	20	9	0	0	0	33	51	32	49	35
PC254K1	18	14	0	0	0	0	29	41	71	59
JL24	18	10	0	0	0	33	58	32	42	35
CG7	16	19	0	0	0	0	59	61	41	39
Kwarts[4]	8	5	0	38	37	0	32	32	31	30
PC287K5	4	8	23	34	0	0	30	21	47	45

[1]Inoculated

[2]Uninoculated

[3]*D. africanus*-susceptible standard

[4]*D. africanus*-tolerant standard

During 2004-2005 the UBS % of inoculated Harts, Sellie, PC254K1 and JL24 were substantially higher than those of their respective uninoculated counterparts. The

UBS % of all the other genotypes did not differ much between the inoculated and uninoculated pairs, although both sections of CG7 were higher than 10 %. Only Kwarts and PC287K5 had UBS % lower than 10 % in this trial (Table 3.6). Also in this trial the grading from choice to crushing corresponded to the respective UBS % (Table 3.6). Choice grade kernels were obtained from the inoculated and the uninoculated sections from PC287K5 as well as from the uninoculated Kwarts. Standard grade was obtained only from Kwarts in the inoculated section and from JL24 and Sellie from the uninoculated section. The rest of the genotypes produced only diverse and crushing grade in the inoculated and the uninoculated sections of the microplot trial.

Differences between yield quantity of each genotype in the uninoculated section and their corresponding inoculated sections ranged from 81 kg/ha (Kwarts) to 2 170 kg/ha (UF85) during 2003-2004 and from 35 kg/ha (JL24) to 1 511 kg/ha (Sellie) during 2004-2005 (Tables 3.7 & 3.8). Yield quantity was variable over and between inoculated and uninoculated sections in both microplot trials (Tables 3.7 & 3.8).

Table 3.7. Yield and income loss or gain of eight groundnut genotypes from inoculated and uninoculated sections of a microplot trial during 2003-2004.

Genotype	Yield (kg/h)		Yield loss/gain	Yield loss/gain (%)	Income (R/ton)		Income loss/gain (%)
	(Yui)	(Yi)	(Yui – Yi)	(Py[1])	(Iui)	(Ii)	(Pin[2])
Sellie[3]	2 420	2 102	318	- 15.1	4 425	2 917	- 51.7
PC223	1 645	2 453	-808	+ 32.9	4 334	2 864	- 51.3
73-30	2 111	1 970	141	- 7.2	4 212	3 742	- 12.6
UF85	593	2 763	-2 170	+ 78.5	3 209	2 889	- 11.1
453	2 699	2 475	224	- 9.1	4 073	3 741	- 8.9
Kwarts[4]	2 653	2 734	-81	+ 3.0	5 394	5 242	- 2.9
PC254K1	988	846	142	- 16.8	4 750	4 801	+ 1.1
PC287K5	1 752	2 675	-923	+ 34.5	3 715	4 354	+ 14.7

[1]Py = (Yui-Yi)100/Yi where Py = yield loss/gain, Yui = yield in uninoculated plots and Yi = yield in inoculated plots.

[2]Pin = (Iui-Ii)100/Ii where Pin = income loss/gain, Iui = income calculated for uninoculated plots and Ii = income calculated for inoculated plots (Grades were priced according to market value at the time of study).

[3] *D. africanus*- susceptible standard

[4]*D. africanus*-tolerant standard

Table 3.8. Yield and income loss or gain of eight groundnut genotypes from inoculated and uninoculated sections of a microplot trial during 2004-2005.

Genotype	Yield (kg/h)		Yield loss/gain	Yield loss/gain (%)	Income (R/ton)		Income loss/gain (%)
	(Yui)	(Yi)	(Yui – Yi)	(Py[1])	(Iui)	(Ii)	(Pin[2])
Kwarts[4]	6 036	5 778	258	-4.4	2 513	1 994	- 26.0
Sellie[3]	5 040	6 551	-1 511	+23.1	1 945	1 710	- 13.7
JL24	5 635	5 600	35	-0.6	1 944	1 777	- 9.4
PC287K5	4 444	4 951	-507	+10.2	2 284	2 094	- 9.1
PC254K1	3 413	2 640	773	-29.3	1 605	1 485	- 8.1
Harts	6 222	6 409	-187	+2.9	1 878	1 855	- 1.2
CG7	5 831	4 382	1 449	-33.1	1 809	1 793	- 0.9
PC299K5	4 160	4 871	-711	+14.6	1 686	1 719	+ 1.9

[1]Py = (Yui-Yi)100/Yi where Py = yield loss/gain, Yui = yield in uninoculated plots and Yi = yield in inoculated plots.

[2]Pin = (Iui-Ii)100/Ii where Pin = income loss/gain, Iui = income calculated for uninoculated plots and Ii = income calculated for inoculated plots (Grades were priced according to market value at the time of study).

[3] *D. africanus*-susceptible standard

[4]*D. africanus*-tolerant standard

During 2003-2004 the yield loss/gain % (Py) ranged from a gain of 78.5 % for UF85 to a loss of 16.8 % for PC254K1 (Table 3.7). Py of the genotypes tested during 2004-2005 ranged from a gain of 23.1 % for Sellie to a loss of 33.1 % for CG7 (Table 3.8).

In contrast to the lower yields produced by Sellie, Kwarts, PC254K1 and PC287K5 during 2003-2004 compared to those produced during 2004-2005, a higher income was earned during 2003-2004 compared to that earned during 2004-2005 (Tables 3.7 & 3.8). A higher income was earned by the genotypes during 2003-2004 because the grading prices (R per ton) for each grading class for this season were much higher than that of 2004-2005 (Landbouweekblad, 2004 & 2005). The differences in income earned for choice grade during 2003-2004 and 2004-2005 were R500 per ton, for standard grade R400 per ton and for crushing and diverse grade R300 per ton each. Only PC287K5 (14.7 %) and PC254K1 (1.1 %) recorded an increase in potential income during 2003-2004 (Table 3.7). During 2004-2005 only PC299K5 showed an increase in potential income (1.9 %). Compared with the decrease in

potential income for Sellie (13.7 %) and Kwarts (26 %) during the same season, the income of CG7 decreased by 0.9 % and that of 254K1 by 8.1 %.

3.4.1.2 Field trials

3.4.1.2.1 Final nematode population densities, reproduction factors and reproduction rate

Nematode populations in the soil from Hartswater and Jan Kempdorp consisted mainly of free-living nematodes and the plant-parasitic spp. *Meloidogyne* and *Helicotylenchus*. At both localities the groundnut genotypes did not differ significantly from each other in terms of the number of either *Meloidogyne* spp. or *Helicotylenchus* spp. per 200 cm^3 soil (data not shown). *Meloidogyne* spp. numbers were low in the roots of the genotypes from both sites. The genotypes did not differ significantly from each other in terms of root-knot nematode numbers per 50 g roots (data not shown).

D. africanus was present in the roots of the groundnut genotypes from the Hartswater trial (Fig. 3.4), but it did not occur in roots of the genotypes from the Jan Kempdorp trial.

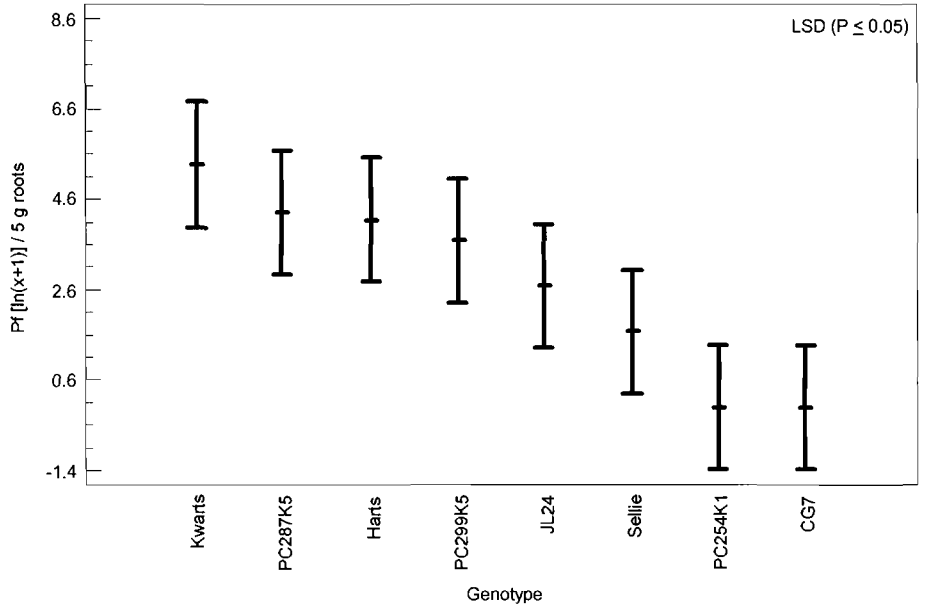

Figure 3.4. *Ditylenchus africanus* numbers (Pf) in the roots of eight groundnut genotypes from a field trial planted at Hartswater during 2004-2005 ($P \leq 0.05$; F-ratio = 4.43).

D. africanus numbers in the roots of CG7 and PC254K1 from Hartswater did not differ significantly from those in the roots of Sellie and JL24, but were significantly lower than those in roots of the other genotypes (Fig. 3.4).

Significantly lower *D. africanus* numbers were extracted from CG7 and PC254K1 pods than from those of the rest of the genotypes planted at both Hartswater and Jan Kempdorp (Figs 3.5 & 3.6). At Jan Kempdorp *D. africanus* numbers of PC287K5 were significantly higher than those in CG7 and PC254K1 but were significantly lower than those in pods of the rest of the genotypes (Fig. 3.6). Pf of *D. africanus* in pods of CG7 was 347.3-fold lower than that of Sellie at Hartswater and 142-fold lower than that of Sellie at Jan Kempdorp. In pods of PC254K1 the Pf was 9.3-fold lower than that of Sellie at Hartswater and 142-fold lower than that of Sellie at Jan Kempdorp.

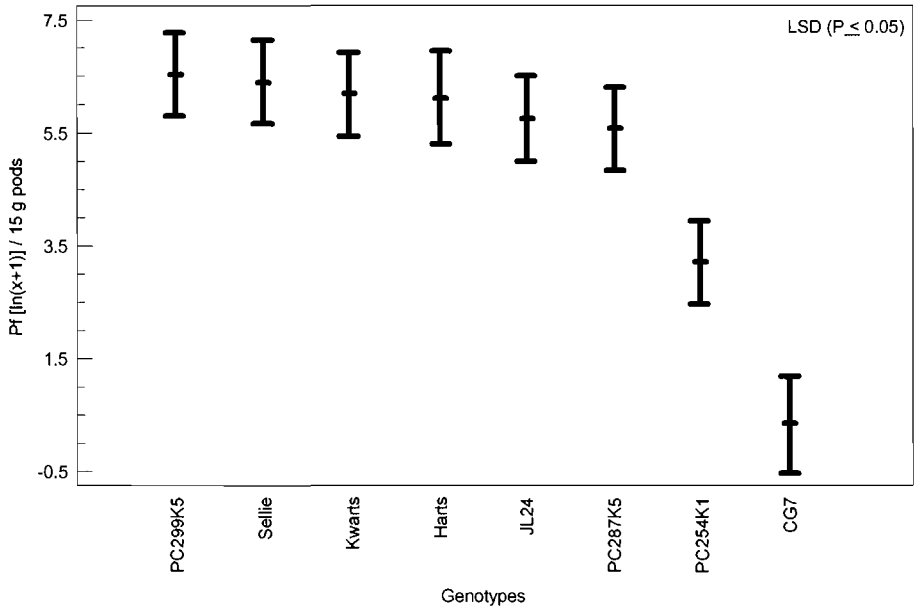

Figure 3.5. *Ditylenchus africanus* numbers (Pf) in pods of eight groundnut genotypes from a field trial planted at Hartswater during 2004-2005 ($P \leq 0.05$; F-ratio = 15.38).

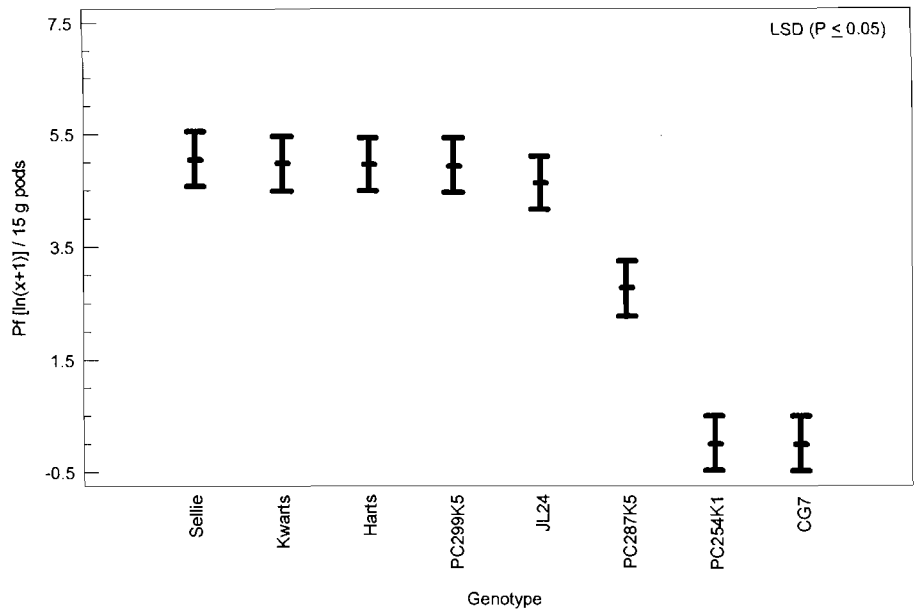

Figure 3.6. *Ditylenchus africanus* numbers (Pf) in pods of eight groundnut genotypes from a field trial planted at Jan Kempdorp during 2004-2005 ($P \leq 0.05$; F-ratio = 44.06).

The numbers of *D. africanus* in pods of Sellie was used as the standard to determine the RR rate of the nematode in pods of the other genotypes. The RR of CG7 was 0 % at both Hartswater and Jan Kempdorp (Table 3.9).

Table 3.9. *Ditylenchus africanus* numbers (Pf) and reproduction rates (RR) in pods of eight groundnut genotypes from field trials planted at Hartswater and Jan Kempdorp during 2004-2005.

Genotype	Pf (15 g pods)		RR[1]	
	Hartswater	Jan Kempdorp	Hartswater	Jan Kempdorp
Sellie[2]	1 042	142	100	100
Kwarts[3]	851	150	81.6	105.6
PC299K5	805	148	77.3	104.2
PC287K5	785	52	75.3	36.6
Harts	371	180	35.6	126.8
JL24	328	105	31.5	73.9
PC254K1	112	0	10.8	0
CG7	3	0	0	0

[1]RR = Pf (genotype) / Pf (Sellie) x 100

[2]*D. africanus*-susceptible standard

[3]*D. africanus*-tolerant standard

RR of PC254K1 was 0 % at Jan Kempdorp and 10.8 % at Hartswater. For PC287K5 the RR was 75.3 % at Hartswater and 36.6 % at Jan Kempdorp.

3.4.1.2.2 Yield assessments

At Hartswater (Table 3.10) the UBS % of Harts was 39 % and that of Sellie was 12 %. The yields of these latter two genotypes were downgraded to diverse and crushing grade. For the rest of the genotypes the UBS % was lower than 10 %. CG7 (4 %), PC287K5 (3 %) and PC254K1 (4 %) had the lowest UBS % and they also produced choice grade kernels. The rest of the genotypes, including the tolerant Kwarts produced standard, diverse or crushing grade.

At Jan Kempdorp (Table 3.11) the UBS % of Harts was 23 % and it was downgraded to diverse and crushing grade. The UBS % of the rest of the genotypes was all below 10 % and all produced choice grade kernels.

Table 3.10. Yield quality of eight groundnut genotypes from a field trial planted at Hartswater during 2004-2005.

Genotype	UBS (%)	Choice (%)	Standard (%)	Diverse (%)	Crushing (%)
Harts	39	0	0	46	54
Sellie[1]	12	0	0	79	21
Kwarts[2]	9	0	53	28	19
JL 24	8	0	59	24	17
PC299K5	7	0	50	35	15
CG7	4	61	0	25	14
PC254K1	4	59	0	27	14
PC287K5	3	31	0	33	36

[1] *D. africanus*-susceptible standard

[2] *D. africanus*-tolerant standard

Table 3.11. Yield quality of eight groundnut genotypes from a field trial planted at Jan Kempdorp during 2004-2005.

Genotype	UBS (%)	Choice (%)	Standard (%)	Diverse (%)	Crushing (%)
Harts	23	0	0	81	19
PC287K5	4	62	0	23	15
Kwarts[1]	3	74	0	20	6
JL 24	3	61	0	31	8
PC299K5	3	55	0	33	12
CG7	3	50	0	36	14
Sellie[2]	1	59	0	34	7
PC254K1	0	63	0	30	7

[1] *D. africanus*-tolerant standard

[2] *D. africanus*-susceptible standard

3.4.2 Reproduction and damage threshold-levels of *D. africanus* on resistant, susceptible and tolerant groundnut genotypes

3.4.2.1 Final nematode population densities and reproduction factor

Pf in pods of PC254K1 did not always increase with an increase in Pi but the Pf of PC287K5 did (Table 3.12). Pf of Sellie and Kwarts also increased with an increase in Pi, except at Pi = 5 000 and higher for Sellie and at Pi = 2 500 and higher for Kwarts.

The RF of Sellie and Kwarts was higher than one at all but the highest Pi's, *viz.* from Pi = 5 000 for Kwarts and Pi = 10 000 for Sellie (Table 3.12). The RF of PC254K1 and PC287K5, however, remained lower than one at all corresponding Pi's.

Table 3.12. Final population densities (Pf) and reproduction factor (RF) of *Ditylenchus africanus* in pods of four groundnut genotypes inoculated at planting with escalating initial population densities (Pi) in a microplot trial during 2004-2005.

Pi	Pf (15 g pods)				RF[1]			
	Sellie[2]	Kwarts[3]	PC254K1	PC287K5	Sellie	Kwarts	PC254K1	PC287K5
0	0	0	0	0	-	-	-	-
50	377	856	16	12	7.54	17.12	0.32	0.24
250	450	366	38	0	1.80	1.46	0.15	0
1 000	2 361	1 228	11	13	2.36	1.23	0.01	0.01
2 500	4 462	2 562	32	58	1.78	1.03	0.01	0.02
5 000	9 586	1 535	83	234	1.91	0.31	0.02	0.05
10 000	6 261	6 146	574	423	0.63	0.62	0.06	0.04
20 000	8 628	4 332	405	671	0.43	0.22	0.02	0.03

[1]RF = Pf / Pi

[2]*D. africanus*-susceptible standard

[3]*D. africanus*-tolerant standard

The relationships between Pi and Pf were best described by non-linear equations for Sellie, Kwarts, PC254K1 and PC287K5 (Fig. 3.7).

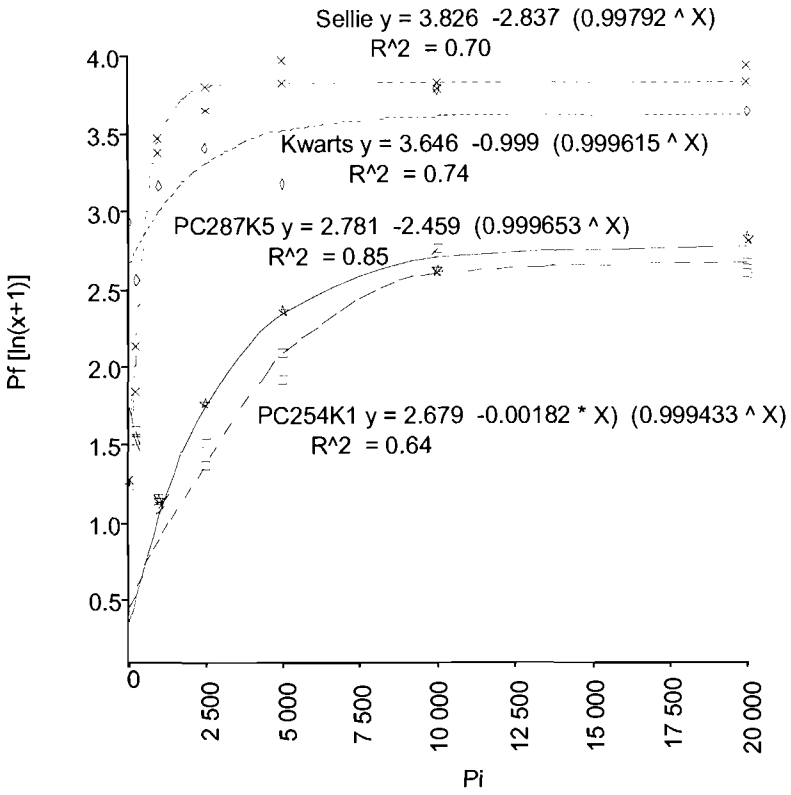

Figure 3.7. Non-linear relationships between initial nematode population densities (Pi) and final nematode population densities (Pf) in pods of Sellie, Kwarts, PC254K1 and PC287K5 in a microplot trial during 2004-2005.

R^2 values were significant for all the genotypes. Exponential models explained 70 % of the variation for Sellie, 85 % for PC287K5, 74 % for Kwarts and 64 % for PC254K1. The regression lines levelled off from Pi = 2 500 for Sellie and Pi = 5 000 for Kwarts but at Pi = 7 500 for PC254K1 and PC287K5.

3.4.2.2 Yield assessments

The UBS % of Sellie was substantially higher than those of the *D. africanus*-tolerant Kwarts and the two breeding lines (Table 3.13). PC287K5 had the lowest overall UBS %. Income from the four genotypes varied over the different Pi levels (Table 3.13).

Table 3.13. Yield quality and income for four groundnut genotypes inoculated at planting with a range of initial *Ditylenchus africanus* population densities (Pi) in a microplot trial during 2004-2005.

Genotype	Pi	UBS (%)	Choice (%)	Standard (%)	Diverse (%)	Crushing (%)	Income (R / ha)
Sellie[1]	0	2	27	0	40	33	4 313
	50	5	0	35	38	27	4 879
	250	6	0	32	40	28	4 178
	1 000	6	0	26	43	31	3 638
	2 500	13	0	0	63	37	3 357
	5 000	20	0	0	65	35	4 184
	10 000	22	0	0	50	50	2 854
	20 000	26	0	0	45	55	2 242
Kwarts[2]	0	3	0	28	38	34	2 807
	50	1	38	0	39	23	5 485
	250	1	31	0	41	28	4 921
	1 000	3	0	34	45	21	4 510
	2 500	5	0	33	45	22	4 194
	5 000	5	0	28	47	25	4 298
	10 000	6	0	26	48	26	3 608
	20 000	11	0	0	65	35	3 292
PC254K1	0	4	0	19	31	50	4 348
	50	2	0	15	30	55	3 980
	250	4	25	0	27	48	3 224
	1 000	2	26	0	33	41	3 335
	2 500	5	0	0	50	50	3 288
	5 000	3	0	20	35	45	4 183
	10 000	3	0	12	20	68	3 362
	20 000	3	0	17	20	63	2 796
PC287K5	0	0.4	34	0	34	32	3 874
	50	0.7	27	0	36	37	4 399
	250	1	40	0	34	28	4 608
	1 000	0.6	26	0	29	45	2 431
	2 500	0.1	32	0	34	34	3 636
	5 000	2	25	0	37	38	3 052
	10 000	1	23	0	35	42	2 874
	20 000	3	0	29	36	35	2 780

[1] *D. africanus*-susceptible standard

[2] *D. africanus*-tolerant standard

The relationships between Pi and UBS % in this trial were best described with a non-linear regression for Sellie (exponential) and linear regressions for PC287K5 and Kwarts. The variation of UBS % for PC254K1 at the different Pf's was not significant and could, therefore, not be fitted to any model. R^2 values were significant for the three former genotypes and the models explained 97 % of the variation for Sellie, 91 % for Kwarts and 63 % for PC287K5 (Fig. 3.8).

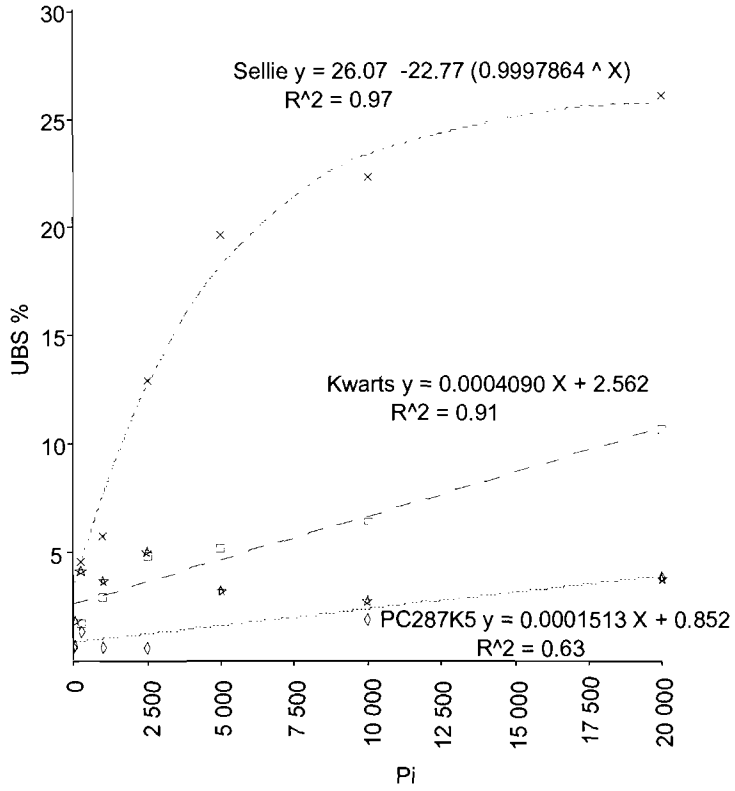

Figure 3.8. Relationships between initial nematode population densities (Pi) and UBS % of Sellie, Kwarts, PC254K1 and PC287K5 in a microplot trial during 2004-2005.

The yield quantities of none of the genotypes corresponded with an increasing Pi (Table 3.14).

Table 3.14. Yield of four groundnut genotypes inoculated at planting with a range of initial *Ditylenchus africanus* population densities (Pi) in a microplot trial during 2004-2005.

Pi	Yield (kg / ha)			
	Sellie[1]	Kwarts[2]	PC254K1	PC287K5
0	1 863	1 446	2 467	1 597
50	2 390	2 116	2 348	1 952
250	2 219	2 030	1 523	1 820
1 000	1 842	2 155	1 505	1 119
2 500	1 835	2 019	1 934	1 540
5 000	2 263	2 114	2 322	1 377
10 000	1 680	1 792	1 872	1 337
20 000	1 356	1 779	1 505	1 442

[1]*D. africanus*-susceptible standard

[2]*D. africanus*-tolerant standard

3.4.3 Expression of *D. africanus* resistance in callus tissue

3.4.3.1 Final nematode population densities and reproduction factor

There were no significant differences between the nematode numbers in Sellie, Kwarts, PC287K5 and PC254K1 callus tissue after four weeks' incubation although *D. africanus* increased from 224- to 393-fold on average over this period (Table 3.12). The RF of *D. africanus* was much higher than one on all callus tissue of the four groundnut genotypes (Table 3.12).

Table 3.15. *Ditylenchus africanus* numbers (Pf) and reproduction factors (RF) on callus tissue of four groundnut genotypes incubated four weeks at 28 °C in a growth cabinet trial during 2008.

Genotype	Pf (1 g callus)	RF[1]
Sellie	3 355	335.5
Kwarts	3 928	392.8
PC287K5	2 240	224.0
PC254K1	2 728	272.8

[1]RF = Pf / Pi

3.5 DISCUSSION

Contrary to the high Pf of *D. africanus* in the susceptible standard Sellie and most of the other genotypes tested during the identification of resistance part of this study, PC254K1 and CG7 pods consistently maintained significantly lower Pf's at harvesting under microplot as well as field conditions. This is fair proof of their resistance (Bos & Parlevliet, 1995) to *D. africanus*. Low Pf in PC287K5 in the microplots also indicated resistance but high Pf in the field shows that the resistance in this line may be lower or less sustainable under field conditions. The superior resistance of PC254K1 and CG7 to *D. africanus* was also evident in the RF because it generally remained below one compared to those of the susceptible standards (Roberts & May, 1986; Windham & Williams, 1988). It was further substantiated by the corresponding RR values that remained below 10 % (Abdel-Momen *et al.*, 1998; Hussey & Janssen, 2002; Timper *et al.*, 2003) for these two lines under microplot as well as field conditions. The field trials in this part of the study provided solid confirmation of the resistance or susceptibility levels of the genotypes tested (De Waele *et al.*, 1989; Venter *et al.*, 1992; Mc Donald, 1998). Although the presence of multiple plant-parasitic nematode species in a field may affect the expression of resistance (Barker & Olthof, 1976; Eisenback, 1985) this did not seem to be applicable to *D. africanus* resistance in PC254K1 and CG7 as indicated in this part of the study.

Increase or decrease in yield quality of the genotypes in all the microplot trials of this part of the study did not correlate with Pf and confirm similar results from previous studies (Venter *et al.*, 1991 & 1992; Mc Donald *et al.*, 2005). Therefore, the much higher yield recorded for UF85 (2003-2004) and Sellie (2004-2005) in the inoculated section of the respective microplot trials compared to that of the uninoculated

sections is difficult to explain. It might in part be due to conjugating environmental factors (Niblack *et al.*, 1986a), which did not seem to be consistent, however.

The higher UBS % of most genotypes in the inoculated sections of the microplot trials compared to the uninoculated sections agrees with several other studies in that the main effect of *D. africanus* on groundnut yield is qualitative (Jones & De Waele, 1988 & 1990; De Waele *et al.*, 1989; Venter *et al.*, 1991 & 1993; Mc Donald *et al.*, 2005). In the presence of aggressive nematodes environmental factors affecting kernel quality (Barker & Noe, 1987; Mc Donald *et al.*, 2005) often play a lesser role in symptom expression (Barker & Noe, 1987). This may also be applicable to *D. africanus* since UBS % of yields obtained from inoculated sections was generally higher compared to the uninoculated counterparts of most genotypes. However, the generally lower UBS % of PC254K1 and CG7 yields compared to those of the other genotypes supports trends in Pf, RF and RR data and confirms the resistance particularly of these two genotypes to *D. africanus* under microplot as well as field conditions.

The superior resistance of PC254K1 to *D. africanus* in the microplot trial on the reproduction and damage potential part of this study was confirmed because Pf did not increase as a function of increasing Pi and remained low even at the highest Pi's (Bos & Parlevliet, 1995). Intra-species competition (Seinhorst, 1965) may have been triggered in PC287K5 when Pi reached 2 500 nematodes per plant and higher but this did not occur in PC254K1 because this genotype did not support reproduction of this nematode even at the lowest Pi levels. Nematode multiplication-threshold levels (Cook & Starr, 2006) were probably reached at Pi = 5 000 and higher for Sellie and at Pi = 2 500 and higher for Kwarts. At these levels the *D. africanus* population increased to such an extent in the pods of the latter two genotypes that resources for further growth and reproduction of the parasite were depleted (Cook & Starr, 2006). Above this multiplication threshold level the Pf of parasites also becomes less than the initial density in a susceptible host (Cook & Starr, 2006). A *D. africanus* multiplication threshold level was not reached in PC287K5 or PC254K1 during this part of the study because the Pf in pods of the latter two genotypes never exceeded the corresponding Pi (Cook & Starr, 2006). High Pf in pods of Sellie and Kwarts at lower Pi's as in previous studies (Basson *et al.*, 1993; Mc Donald *et al.*, 2005) showed that survival of *D. africanus* in either small or large numbers at the beginning of a growing season is irrelevant because of the nematode's high reproduction potential (De Waele & Wilken, 1990). The resistance of PC254K1 to *D. africanus* was

furthermore substantiated by the RF, which remained below one (Roberts & May, 1986; Windham & Williams, 1988) at all Pi levels. Based on RF > 1 the nematode was able to reproduce sufficiently on Sellie and Kwarts at lower Pi levels, however (Windham & Williams, 1988). The non-linear relationship between Pf and UBS % for Sellie in the trial on reproduction and damage-threshold levels is similar to that observed previously in microplot studies, which propose that UBS % is a reliable estimate of the effect of this nematode (Mc Donald *et al.*, 2005). Compared with Sellie and Kwarts the resistance of PC254K1 to *D. africanus* is, therefore, also confirmed by its high yield quality, having produced only choice and standard grade kernels at all Pi levels.

The high Pf and RF of *D. africanus* on callus tissue of Sellie, Kwarts, PC287K5 as well as on those of PC254K1 confirms that *D. africanus* is able to reproduce optimally on callus tissue of all four groundnut genotypes (Windham & Williams, 1988). These results support previous studies, which indicated that callus tissue of plants resistant to a specific nematode in nature will often support good reproduction of that nematode on callus tissue of the same resistant plant (Webster & Lowe, 1966; Krusberg & Babineau, 1977). The higher reproduction rate of nematodes on callus tissues compared to that on resistant hosts has previously been reported (Krusberg & Babineau, 1977). According to Webster and Lowe (1966), plant growth-regulating substances such as 2.4-D used in the medium on which the callus tissue was cultured during this study (Van der Walt & De Waele, 1989) may have played a role in making incompatible cells compatible to nematodes in callus tissue (Webster & Lowe, 1966). Nematode resistance of groundnut genotypes can, therefore, not be evaluated against *D. africanus* based on Pf and RR obtained from their callus tissue. This part of the study did, however, confirm the high reproduction rate of *D. africanus* in *in vitro* callus tissue from groundnut leaves that was previously reported by Van der Walt and De Waele (1989) in their studies.

This is the first report of groundnut genotypes that express high-level, sustainable resistance to *D. africanus* under microplot as well as field conditions. PC254K1 will play an important role in groundnut-breeding programmes because of its evidently high level of resistance, even at high nematode infestation levels. Although this line may lack many desirable traits required for an agronomically acceptable cultivar e.g. high yield potential, desired kernel size, colour and form it should be acceptable for use to introgress resistance into preferred breeding material. A high-yielding cultivar

developed from PC254K1 should also be able to produce better kernel quality, which may increase the net income per ha (Mc Donald *et al.*, 2005).

Although only tested in one part of this study, there are strong indications that CG7 may also have superior resistance to *D. africanus*. It warrants further investigation should a comprehensive programme on the introgression of nematode resistance in groundnut be initiated.

Although PC287K5 also maintained low nematode numbers in some trials, its level of resistance does not seem to be as high as that of PC254K1. However, it still performed well compared to tolerant cultivar Kwarts, which might indicate that this line could also be tolerant. Therefore, it may still play an important role, particularly under lower nematode population pressure.

CHAPTER 4

REPRODUCTIVE AND DAMAGE POTENTIAL OF FIVE GEOGRAPHICALLY-ISOLATED *DITYLENCHUS AFRICANUS* POPULATIONS ON GROUNDNUT

4.1 INTRODUCTION

From an economic and environmental perspective host-plant resistance is one of the most preferred management tools for plant-parasitic nematodes on a variety of crops (Starr *et al.*, 2002; Agudelo *et al.*, 2005; Dickson & De Waele, 2005; Cook & Starr, 2006). This also applies to the management of *D. africanus* on groundnut (De Waele *et al.*, 1990). The breeding line PC254K1 was confirmed to be highly resistant to *D. africanus* (Chapter 3) and showed potential for inclusion in the local groundnut-breeding programme as a primary source of resistance.

Acceptance of a new cultivar developed from PC254K1 on the market will not only depend on the agronomic acceptability of such a cultivar, but also on the sustainability of its resistance to *D. africanus*. Sustainability of resistance may in turn depend on the virulence and damage potential of nematode populations present in soils where the crop is produced (Blok *et al.*, 1997; Thies & Ferry, 2002). Potential differences in reproduction and damage potential of geographically-isolated *D. africanus* populations will, furthermore, affect the composition of a suitable management system for a specific production area (Sree Latha *et al.*, 1989; Noe, 1992). Obviously the usefulness of a specific resistant cultivar for each area is also dependent on the damage potential of a specific plant-parasitic nematode for a particular area. Knowledge of any variability between and within *D. africanus* populations present in the various groundnut production areas is, therefore, necessary for development of cultivars of which their resistance will be sustainable for all respective areas of production (Agudelo *et al.*, 2005).

Although a number of studies have been done on the reproduction of *D. africanus* on groundnut callus tissue (Van der Walt & De Waele, 1989) at temperatures that ranged from 16 °C to 34 °C (De Waele & Wilken, 1990) and on the reproduction and damage potential of *D. africanus* on a number of groundnut genotypes (Basson *et al.*, 1991, 1992 & 1993; Venter *et al.*, 1991 & 1993; Van der Merwe & Joubert, 1994; Mc Donald *et al.*, 2005), no information is available on the comparative reproduction and damage potentials of *D. africanus* populations isolated from different geographical

locations. The purpose of this study was to establish under controlled and semi-controlled conditions whether there are differences in the reproduction and damage potential of *D. africanus* originating from different localities in the groundnut-producing areas of South Africa.

4.2 MATERIALS AND METHODS

4.2.1 *D. africanus* populations

Callus used for propogation of the various geographically-isolated *D. africanus* populations was initiated from young, surface-sterilised leaves of Sellie (Chapter 2). *D. africanus* populations used for the inoculation of callus tissue were extracted from infected groundnut pods that were collected from infested fields from Mareetsane (26.15°S, 25.43°E) (population 1), Jan Kempdorp (27.95°S, 24.85°E) (population 2), Vaalharts (27.83°S, 24.79°E) (population 3), Schweizer-Renecke (27.19°S, 25.33°E) (population 4) and Theunissen (28.40°S, 26.71°E) (population 5) (Fig. 4.1).

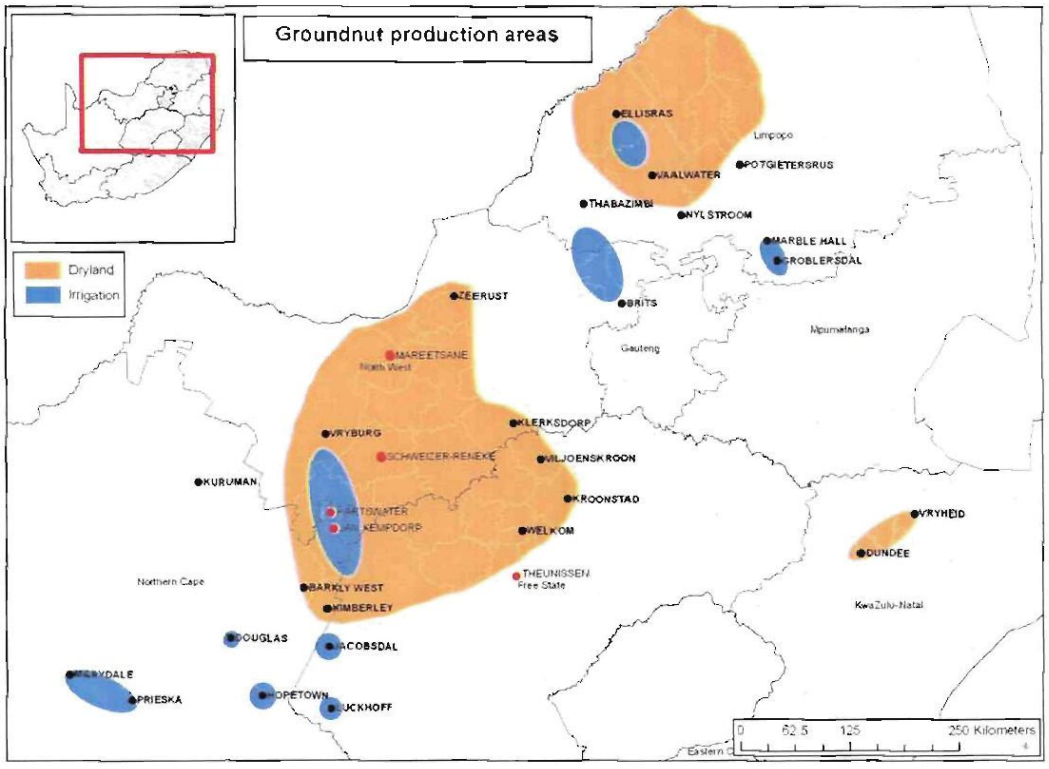

Figure 4.1. *Ditylenchus africanus* populations were isolated from infected groundnut pods collected from five different localities (indicated by red dots) within the groundnut-production area of South Africa.

These infected pods were collected from groundnut shelling plants at the abovementioned locations by officers of the Perishable Products Export Control Board (PPECB) of South Africa. The material was delivered to the Nematology Unit of ARC-GCI in Potchefstroom within two to three days of collection. After collection the pods were put in dry cooler bags and were stored at room temperature under air-dry conditions until extraction of the nematodes. Twenty pods from each locality were shelled by hand and a mean number of 251 484 *D. africanus* was extracted from the pod tissues (5 g hull and 5 g kernel) from each locality using the soaking method (Chapter 2). *In vitro*, aseptic calluses were then established of each of the five above mentioned *D. africanus* populations with five males and five females picked from the extracted *D. africanus* pod-samples following procedures described in Chapter 2.

4.2.2 Trial layout

4.2.2.1 Growth cabinet trial

This trial was conducted during July / August 2007. Three growth cabinets were set at 21 °C, 28 °C and 35 °C, respectively, and allowed to stabilise for three days at the respective temperature before proceeding with this trial. Ninety calluses of $\pm$ 1.48 g each were used. Nematodes inoculated on the callus were extracted from cultures established for each locality (4.2.1). Each of the ninety calluses was inoculated with five males and five females of the respective *D. africanus* populations following the relevant procedures described in Chapter 2.

The trial was laid out in randomised complete block, factorial design. The three growth cabinets with the different temperature regimes were the main factor. The sub-factor consisted of callus tissue cultures of each of the five *D. africanus* populations (five treatments), each replicated six times. The inoculated calluses were incubated in darkness for four weeks in the growth cabinets to allow the nematodes to complete a minimum of four life cycles at a specific temperature regime (De Waele & Wilken, 1990). After this the nematodes were extracted from the callus tissue according to the procedures described in Chapter 2. The nematodes from each callus were counted under a research microscope and the nematode (Pf) numbers were expressed as *D. africanus* per 1 g callus.

4.2.2.2 Greenhouse trial

This trial was conducted between May and October 2007. Sixty plastic pots were filled with 4 000 cm^3 sandy-loam soil fumigated with EDB six weeks before planting (Chapter 2). Nutrients were added and mixed into the soil in each pot as desribed in Chapter 2. Nutrient requirements for this trial were 5.84 g dolomitic lime, 3.52 g super phosphate (10.5 % P), 2.32 g sodium chloride (KCl) and 1.50 g calsium nitrate ($CaNO_3$) per pot. One seed was planted to a depth of 5 cm per pot (Chapter 2). A plastic marker per pot indicated the genotype and replicate numbers. Sellie and PC254K1 seeds were obtained from nematode-free mircoplot nurseries (Chapter 2) and treated with fungicide and nitrogen-fixing bacteria as described in Chapter 2.

The trial was planted in a randomised complete split-plot design in a greenhouse with a temperature regime of 18 °C to 27 °C with a 13-h photoperiod. Factor one was a plot of 30 pots each of Sellie (suscepitble) or PC254K1 (resistant). The trial included two factors and six single-plant replicates inoculated with each of the five *D. africanus* populations. Each pot was inoculated at planting with ± 2 000 *D. africanus* consisting of various life stages. The nematodes used for inoculation were extracted from *in vitro* cultures of the five *D. africanus* populations (4.2.1) and prepared for inoculation according to procedures described in Chapter 2. A plastic marker per pot indicated the genotype, *D. africanus* population and replicate number. Pots were rotated clockwise every fortnight to limit the effects of temperature or air-flow gradients in the greenhouse. Pots were watered three times a week by hand.

Nematode assessments were made at harvesting on all the plants. *D. africanus* was extracted from pegs, hulls and kernels following the respective methods described in Chapter 2. Nematodes were counted under a research microscope and expressed as Pf per 15 g pods (Chapter 2). Yield assessments were not made from the single-plant pots in the greenhouse trial because the technique used for nematode extraction is destructive (Bolton *et al.*, 1990) and the amount of kernels left for reliable yield assessments was insufficient.

4.2.2.3 Microplot trial

This trial was conducted during 2007-2008 in concrete-pipe microplots (Chapter 2). All sixty plots were filled with the same fumigated sandy-loam soil used in the greenhouse trial (4.2.2.2). Nutrients incorporated into the soil of each plot were 70 g

dolomitic lime, 51 g super phosphate (10.5 % P), 34 g KCl and 20 g $CaNO_3$. Each plot was then planted with six seeds respectively of Sellie or PC254K1 to a depth of 5 cm (Chapter 2). Seeds of the two genotypes were obtained from nematode-free nurseries, treated with fungicide and inoculated with *Bradyrhizobium arachis* before planting as described in Chapter 2.

The trial was planted in a randomised complete split-plot design similar to that of the greenhouse trial (4.2.2.2) with two factors, five treatments and six replicates each. Factor one consisted of 30 plots of six plants each of Sellie and 30 plots of six plants each of PC254K1. The inoculum density was $\pm$ 2 000 *D. africanus* of various life stages per plant at planting. *D. africanus* used for inoculation of the plants were extracted from the same *in vitro* cultures of the five geographically-isolated *D. africanus* populations (factor two: treatments) used in the greenhouse trial (4.2.2.2). Every treatment was replicated six times. Preparation of *D. africanus* inoculum and inoculation of each plant was performed as described in Chapter 2. A plastic marker indicated the genotype, *D. africanus* population and the replicate number of each plot. Supplementary to rainfall plots were irrigated three times a week through a micro-sprayer placed in the centre of each plot (Chapter 2).

Nematode and damage assessments were done at harvesting. Separate nematode extractions were done from peg, hull and kernel samples collected from two randomly picked plants from each plot. *D. africanus* was extracted following the methods described in Chapter 2. Nematodes were counted under a research microscope and expressed as Pf per 15 g pods (Chapter 2).

The four remaining plants in each plot not used for nematode extraction were used for damage assessments. Yield quantity was not determined for this part of the study because *D. africanus* does not affect yield quantity (Chapter 3; Venter *et al.*, 1991 & 1992; Mc Donald *et al.*, 2005). Yield quality was determined by following standard grading procedures (Chapter 2).

4.3 STATISTICAL ANALYSES

Unless otherwise stated nematode data were ln(x+1) transformed before being subjected to a factorial analysis of variance (ANOVA) (Stat Graphics 5 Plus for Windows). Means were separated by a LSD test ($P \leq 0.05$).

Nematode variables included final population densities (Pf) of *D. africanus* present in callus tissue cultures in the growth cabinet trial and Pf present in pods of Sellie and PC254K1 in the greenhouse and microplot trials. The nematode reproduction factor (RF) in callus tissue cultures (growth cabinet trial) and pods of Sellie and PC254K1 (greenhouse and microplot trials) were determined as described in Chapter 2.

4.4 RESULTS

4.4.1 Growth cabinet trial

4.4.1.1 Final nematode population densities and reproduction factor

There was no significant interaction between temperature regime and *D. africanus* population numbers. However, there were significant differences between the populations at 21 °C as well as at 28 °C (Fig. 4.2). In the 21-°C regime the Pf of population 4 (Schweizer-Renecke) was significantly higher than that of population 1 (Mareetsane), 2 (Jan Kempdorp) and 5 (Theunissen) (Fig. 4.2).

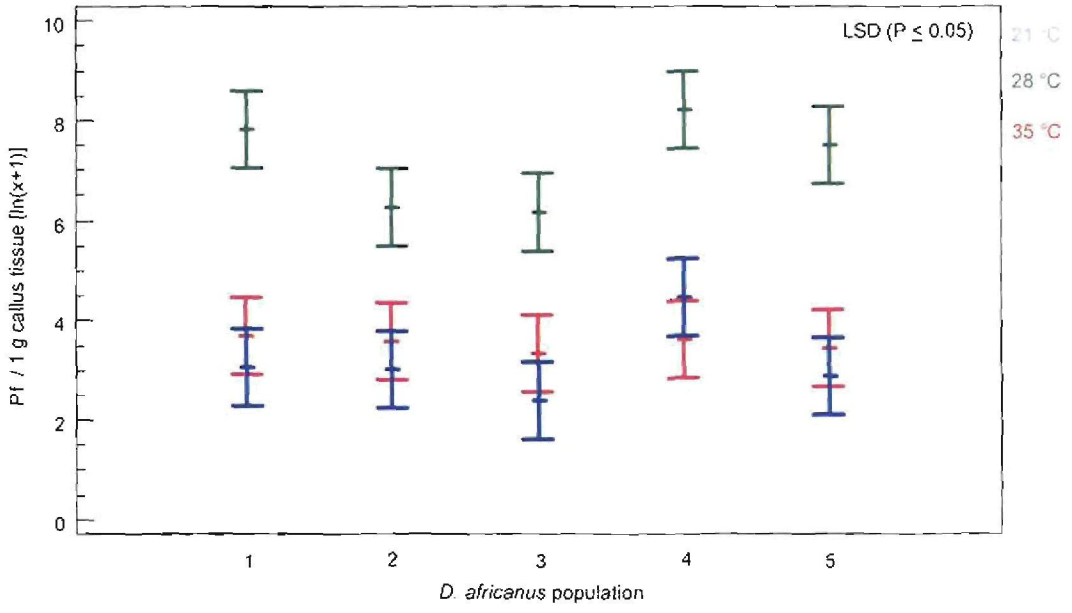

Figure 4.2. Final *D. africanus* numbers (Pf) on groundnut callus tissue inoculated with five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) incubated for four weeks at 21 °C, 28 °C or 35 °C in three separate growth cabinets during 2007 ($P \leq 0.05$; F-ratio = 4.21).

Populations 1, 2, 3 (Vaalharts) and 5 did not differ significantly from each other. In the 28-°C regime the Pf of population 4 was significantly higher than that of populations 2 and 3. No significant differences existed between the Pf of the five populations at the 35-°C regime.

Significant differences existed in terms of the Pf of the five populations when it was pooled over each of the three temperature regimes (Fig. 4.3). Pf of the populations pooled over 28 °C were significantly higher than those pooled over 21 °C and 35 °C. However, Pf of the populations on callus tissue pooled over 21 °C and 35 °C did not differ significantly from each other.

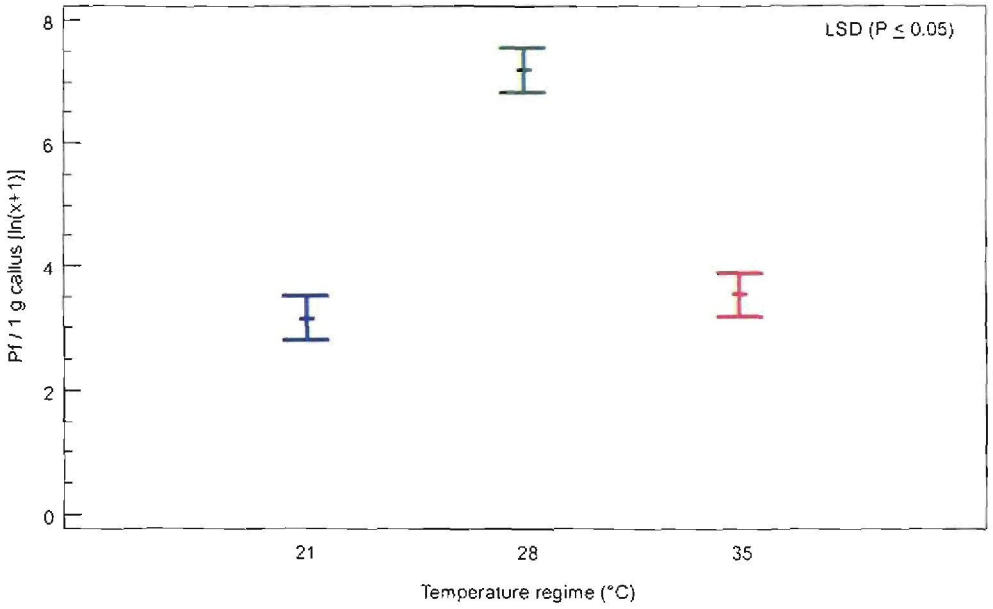

Figure 4.3. Final *D. africanus* numbers (Pf) on groundnut callus tissue inoculated with five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) pooled over three temperature regimes, respectively, in a growth cabinet trial during 2007 ($P \leq 0.05$; F-ratio = 76.69).

The RF of all five populations at all three temperature regimes was higher than one (Table 4.1). The RF of the populations at 28 °C were ± 58- to 373-fold higher than those of the same populations on callus tissues at 21 °C and ± 35- to 156- fold higher than those of the same populations at 35 °C. The RF rates at 35 °C were on average only slightly higher than those at 21 °C.

Table 4.1. Reproduction factor (RF) of five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) on groundnut callus incubated four weeks at 21 °C, 28 °C and 35 °C in separate growth cabinets during 2007.

Nematode	RF[1]		
population	21 °C	28 °C	35 °C
4	11.0	1 286.7	8.6
1	3.4	650.8	5.1
2	3.0	174.0	4.6
5	2.3	856.8	5.5
3	1.5	126.3	3.6
Mean	4.2	587.5	5.5

[1]RF = Pf / Pi

4.4.2 Greenhouse trial

4.4.2.1 Final nematode population densities and reproduction factor

A significant interaction existed between the groundnut genotypes and the Pf of the five *D. africanus* populations in the greenhouse trial. Pf of all five populations was significantly higher in Sellie than in PC254K1 (Fig. 4.4). No significant differences existed between the five populations on Sellie. There were significant differences in Pf of the populations in PC254K1, however (Fig. 4.4). Pf of population 2 (Jan Kempdorp) was significantly lower than those of the rest.

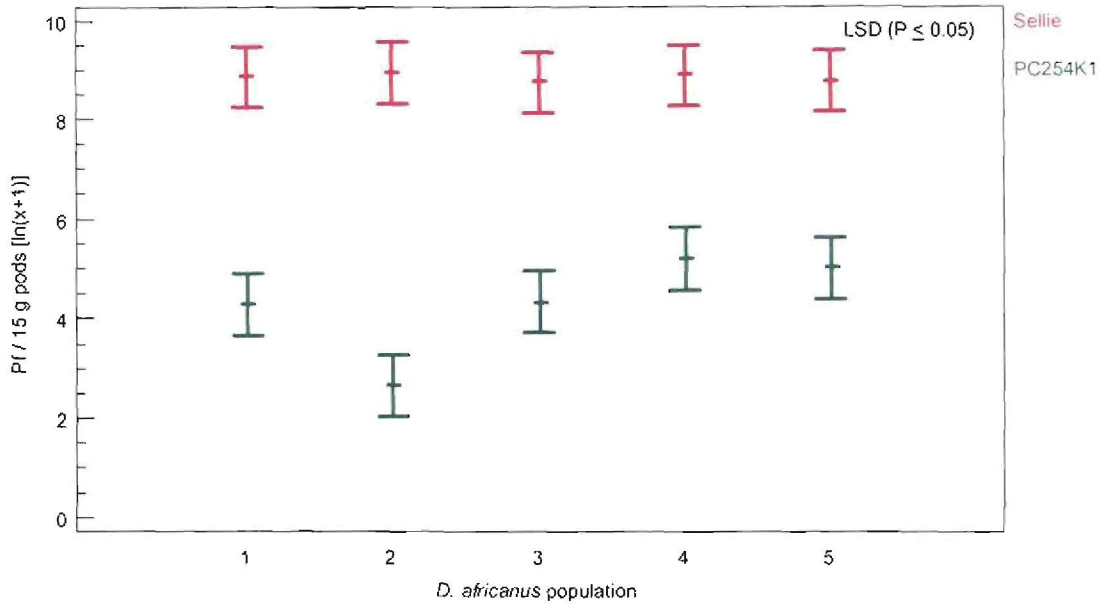

Figure 4.4. Final *Ditylenchus africanus* numbers (Pf) in pods of Sellie and PC254K1 inoculated at planting with five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) in a greenhouse trial during 2007 ($P \leq 0.05$; F-ratio = 2.92).

The RF of all five populations on Sellie were higher than one (Table 4.2). In contrast to this the RF on PC254K1 were all lower than one. The reproduction of the five populations was on average ± 51-fold lower on PC254K1 than on Sellie.

Table 4.2. Reproduction factors (Pf) of five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) in pods of Sellie and PC254K1 in a greenhouse trial during 2007.

Nematode	RF[1]	
population	Sellie	PC254K1
2	3.94	0.02
4	3.82	0.10
1	3.59	0.09
5	3.25	0.11
3	3.22	0.05
Mean	3.56	0.07

[1]RF = Pf / Pi

4.4.3 Microplot trial

4.4.3.1 Final nematode population densities and reproduction factor

No significant interaction existed between the five populations and the two groundnut genotypes. Pf of the populations on Sellie and PC254K1 were relatively low for all five populations (Fig. 4.5). Although the Pf of all of the corresponding nematode populations were higher in Sellie than in PC254K1 there were no significant differences between the respective populations in either genotype.

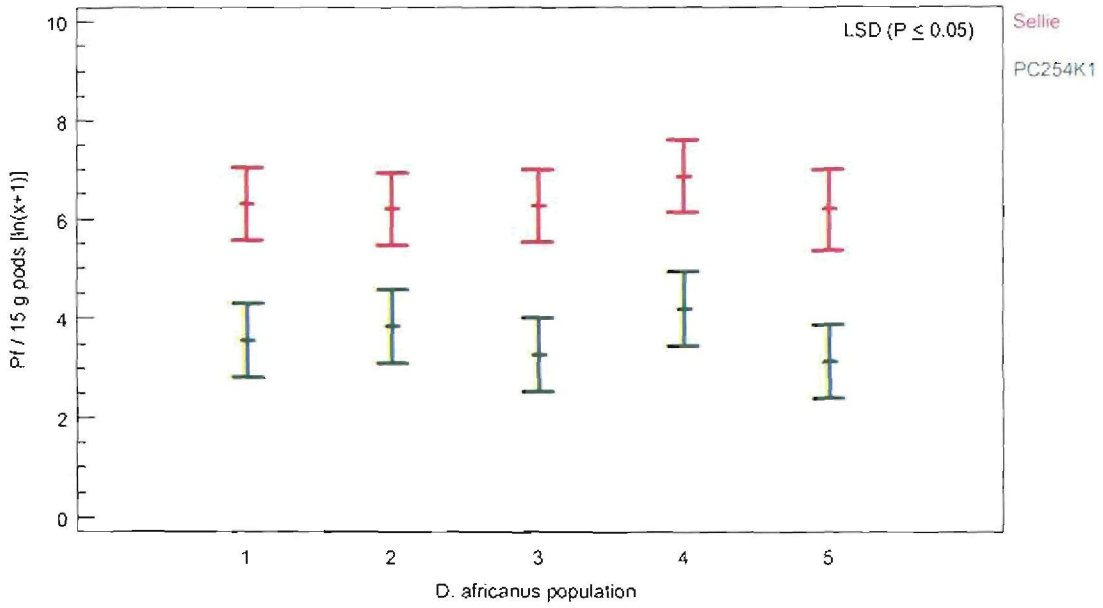

Figure 4.5. Final *Ditylenchus africanus* numbers (Pf) in pods of Sellie and PC254K1 inoculated at planting with five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) in a microplot trial during 2007-2008 ($P \leq 0.05$; F-ratio = 75.76).

The low Pf of the five populations in the pods of Sellie and PC254K1 were also reflected in their respective low RF, which remained lower than one for both genotypes (Table 4.3).

Table 4.3. Reproduction factor (Pf) of five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) in pods of Sellie and PC254K1 in a microplot trial during 2007-2008.

Nematode	RF^2	
population	Sellie	PC254K1
4	0.68	0.03
1	0.51	0.08
3	0.29	0.02
2	0.27	0.03
5	0.25	0.02
Mean	0.40	0.04

[1]RF = Pf / Pi

In spite of the low Pf, however, the average reproduction on PC254K1 was still only one tenth of that of Sellie.

4.4.3.2 Damage assessments

UBS % of PC254K1 varied and ranged from 4 % (population 3) to 9 % (populations 4 and 5) (Table 4.4). PC254K1 produced choice grade (population 3) or standard grade (populations 1, 2, 4 and 5). In contrast with the UBS % of PC254K1 kernels those of Sellie were all higher than 10 %, ranging from 12 % to 14 %. Sellie kernels graded crushing and diverse grade.

Table 4.4. Yield quality of Sellie and PC254K1 inoculated at planting with five geographically-isolated *Ditylenchus africanus* populations from Mareetsane (population 1), Jan Kempdorp (population 2), Vaalharts (population 3), Schweizer-Renecke (population 4) and Theunissen (population 5) in a microplot trial during 2007-2008.

Genotype	Nematode population	UBS (%)	Choice (%)	Standard (%)	Diverse (%)	Crushing (%)
Sellie	1	14	0	0	61	39
	5	12	0	0	64	36
	2	13	0	0	70	30
	4	12	0	0	58	42
	3	13	0	0	41	59
PC254K1	4	9	0	41	19	40
	3	4	35	0	21	44
	1	8	0	42	21	37
	2	7	0	33	18	49
	5	9	0	36	19	45

4.5 DISCUSSION

No significant interaction between temperature regimes and Pf of the five different *D. africanus* populations in the growth cabinet trial implies that the increase in population at the different temperature regimes was similar for all. However, the data of this trial showed that temperature regime has a great effect on *D. africanus* population growth and that 28 °C is close to the optimum temperature for reproduction of this nematode (De Waele & Wilken, 1990) for a variety of different populations. The lower Pf at 21 °C and at 35 °C compared to that of 28 °C may be attributed to a reduction in egg production and development (De Waele & Wilken, 1990).

In the greenhouse trial the respective *D. africanus* populations behaved differently on Sellie than on PC254K1. Although the latter genotype consistently showed resistance to all the *D. africanus* populations its expression of resistance against population 2 (Jan Kempdorp) was significantly stronger than against the rest of the populations. The lack of significant interaction between the two groundnut genotypes and the five *D. africanus* populations implies that the increase of the *D. africanus* populations on each of the two groundnut genotypes were similar. In contrast to the greenhouse

trial, the five *D. africanus* populations did not behave differently in terms of reproduction rate on Sellie and PC254K1 in the microplots since no significant interaction existed between the two groundnut genotypes and the five populations. The lower nematode numbers from Sellie in the microplot trial compared to that extracted from Sellie from the greenhouse trial has previously been reported (Venter *et al.*, 1991; Mc Donald *et al.*, 2005). However, the low Pf of the nematodes extracted from pods of PC254K1 in the greenhouse and microplot trial confirmed PC254K1's resistance to all five *D. africanus* populations. Resistance of a host plant is determined, among others by its effect on nematode reproduction (Trudgill, 1986) that could be defined as the ability to inhibit the reproduction of a population relative to the reproduction of the population on the susceptible host (Cook & Evans, 1987; Roberts, 2002).

Although the RF of the *D. africanus* populations on groundnut callus tissue at 28 °C in the growth cabinet trial was much higher than those at 21 °C and 35 °C the RF, which was also higher than one at the latter two temperature regimes support findings of De Waele and Wilken (1990) that egg viability and start of egg hatching at these temperatures may be similar to those at 28 °C. This data further indicate that all the *D. africanus* populations were still able to increase at the latter two temperatures even though their reproduction rate was not optimal. The resistance of PC254K1 to *D. africanus* was again confirmed by RF in the greenhouse as well as in the microplots (Roberts & May, 1986; Windham & Williams, 1988).

The small range in UBS % between the five different populations in Sellie indicates that the damage potential of the populations on groundnut is similar. Mc Donald *et al.* (2005) showed strong relationships between the presence of *D. africanus* and UBS %. Venter *et al.* (1991) also provided strong evidence that UBS % is a reliable indication of *D. africanus* damage. The low UBS % of PC254K1 compared to that of Sellie is, therefore, further confirmation of its resistance to all five *D. africanus* populations. The differences, though being small, between the UBS % of the populations do, however, not agree with differences in Pf or RF of either Sellie (susceptible) or PC254K1 (resistant).

The results of this study indicated that the reproduction and damage potential of the five *D. africanus* populations representative of the local groundnut-production area were similar. The near optimum temperature for development of *D. africanus* populations is 28 °C but they are able to reproduce (although not optimally) over a

wide range of temperature regimes. Resistance of PC254K1 to all five *D. africanus* populations was confirmed in all nematode and plant variables in this study. The results suggest that the resistant trait of a *D. africanus* resistant cultivar developed from PC254K1 should be sustainable over the whole groundnut production area.

CHAPTER 5

THE MECHANISM OF RESISTANCE TO *DITYLENCHUS AFRICANUS* EXPRESSED BY THE GROUNDNUT GENOTYPE PC254K1

5.1 INTRODUCTION

The success of strategies used in long-term nematode management programmes will depend on the understanding of the reaction of the host plant to attack by plant-parasitic nematode populations, including the mechanism of resistance involved (Kotcon *et al.*, 1987). The histopathology of *D. africanus* has previously been studied on the cultivar Sellie, which is highly susceptible to this nematode (Jones & De Waele, 1990; Venter *et al.*, 1995). Resistance to this nematode has been identified for the first time during this study (Chapter 3). Therefore, no information is available on the histopathology of this nematode on resistant genotypes. The objective of this study was to study the histopathology of *D. africanus* on PC254K1 and compare it to that of Sellie in order to determine the mechanism of resistance involved.

5.2 MATERIALS AND METHODS

PC254K1 and Sellie material for the histological study was propagated in a greenhouse under conditions of optimum growth for the genotypes and those that allowed uninhibited development and feeding of *D. africanus* on its host plant. These plants were propagated in the greenhouse from May to October 2008. The microscope slides prepared from the PC254K1 and Sellie material were studied under a light microscope.

5.2.1 Trial layout in the greenhouse

Twenty-eight plastic pots were filled with 4 000 cm^3 fumigated, sandy-loam soil as described in Chapter 2. Nutrients mixed into the soil (Chapter 2) included 5.80 g dolomitic lime, 3.45 g super phosphate (10.5 % P), 2.25 g sodium chloride (KCl) and 1.60 g calcium nitrate ($CaNO_3$) per pot. Seeds were obtained from nematode-free mircoplot nurseries (Chapter 2) and treated before planting with fungicide and nitrogen-fixing bacteria following the procedures described in Chapter 2. One seed was planted per pot to a depth of 5 cm (Chapter 2).

The trial was done in a greenhouse with a temperature regime of 18 °C to 27 °C and a 13-h photoperiod. Six pots each of Sellie and PC254K1 were inoculated at planting with 0 (nematode-free), 2 000, 5 000 or 7 000 *D. africanus* of various life stages. Nematodes for inoculation were obtained from an *in vitro* culture of the Vaalharts population (Chapters 2 & 3) of *D. africanus* according to the procedures described in Chapter 2. A plastic marker in each pot indicated the groundnut genotype, nematode inoculum level and replicate number. The pots were rotated clockwise every fortnight to eliminate the effect of temperature or light gradients in the greenhouse. The plants were watered by hand three times a week.

Pegs not yet developed into mature pods as well as mature pods were collected at 90 days after plant (DAP), 120 DAP and at harvesting (150 DAP). At each sampling time two plants from each genotype and inoculum level were separated from the soil (Chapter 2), their pegs and pods removed (Chapter 2), mixed by hand and ten randomly-picked pegs and pods were collected from each replicate. Pods collected at 150 DAP were photographed to compare external *D. africanus* damage symptoms between the two genotypes before fixing the material in paraformaldehyde. This was done only at 150 DAP because this is the period when *D. africanus* populations peak in the pods (Basson *et al.*, 1990). External damage caused by these nematodes is, therefore, supposed to be optimal at 150 DAP. The rest of the plant tissue not used for histopathology studies was discarded. The material was washed free of soil under a gentle stream of running tap water and fixed in 4 % paraformaldehyde contained in plastic 250-ml beakers. The material was stored at room temperature for a maximum of three days before preparation for light-microscope studies commenced.

5.2.2 Preparation of plant tissue for light-microscope studies

The plant material was prepared at room temperature under a flow cabinet. Pieces of peg and pod tissue were cut in approximately 2-mm sections and fixed in Todd's fixative (pH 7.5) in 20-ml glass flasks for 2 h (Todd, 1986). After 2 h the Todd's fixative was carefully removed with a fine glass pipette and replaced with 0.05-M sodium cacodilate buffer in which the tissue samples were impregnated for 15 min. This process was repeated three more times. The material was post-fixed in 1-% osmium tetroxide for 1 h and then washed in three changes of distilled water for 15 min each. The material was then dehydrated in an ethanol series of 50 %, 70 %, 90 % and 100 % for 15 min each. The 100-% ethanol was then carefully poured off and replaced with 100-% LR White resin in which the material was allowed to be

impregnated for 10 min. After that the resin was carefully poured off and replaced with fresh resin, leaving it for 1 h. The resin was then again carefully poured off and replaced with fresh resin in which the plant material was left overnight at 4 °C in a refrigerator. The next day the material was finally embedded in fresh resin contained in marked gelatine capsules, which were cured overnight in an oven at 60 °C (O'Brien & Mc Culley, 1981). One-µm sections (longitudinal and cross sections) of the plant material were cut with glass knives on a rotary microtome, floated on microscope slides and air-dried. The material on each microscope slide was stained with 0.5-% aqeous toluidine blue (Lillie & Fullmer, 1976) and 0.05-% neofuchsin for 3 min. The remainder of the stain was rinsed off with tap water and the stained plant material allowed to dry for 3 min before it was covered with a cover slide. At each sampling interval (90, 120 and 150 DAP) 20 microscope slides consisting of 10 slides of peg tissue and 10 slides of pod tissue were prepared and examined for each genotype at each *D. africanus* inoculum level. Light micrographs were taken from selected slides. Orientation of the material on the microscope slide and identification of the different structures were done with the assistance of trained personnel of the School of Environmental Sciences and Development as well as the Electron microscopy unit at North-West University, Potchefstroom.

5.3 RESULTS

5.3.1 External damage symptoms on mature pods collected at 150 DAP

At 150 DAP damage symptoms were visible on pods of Sellie inoculated with *ca.* 2 000, 5 000 as well as 7 000 *D. africanus* specimens. Damage symptoms on Sellie and PC254K1 pods were most severe at the 7 000-nematodes inoculum level (Fig. 5.1). Upon inspection of Sellie pods the tissue at the connection of the peg to the pod appeared brown and necrotic. The veins that extended longitudinally in the exocarp just beneath the surface of the pods had a brown discoloration similar to that of the tissue at the peg-pod connection. On some pods of Sellie this dark discoloration extended over a large portion of the pod surface. At 150 DAP pods of PC254K1 at an inoculum level of 7 000 *D. africanus* specimens were not discolored at the peg-pod connection point or over the pod surface (Fig. 5.1). No discoloration of the peg-pod connection or the pod surface was visible on Sellie or PC254K1 from the nematode-free control at 150 DAP.

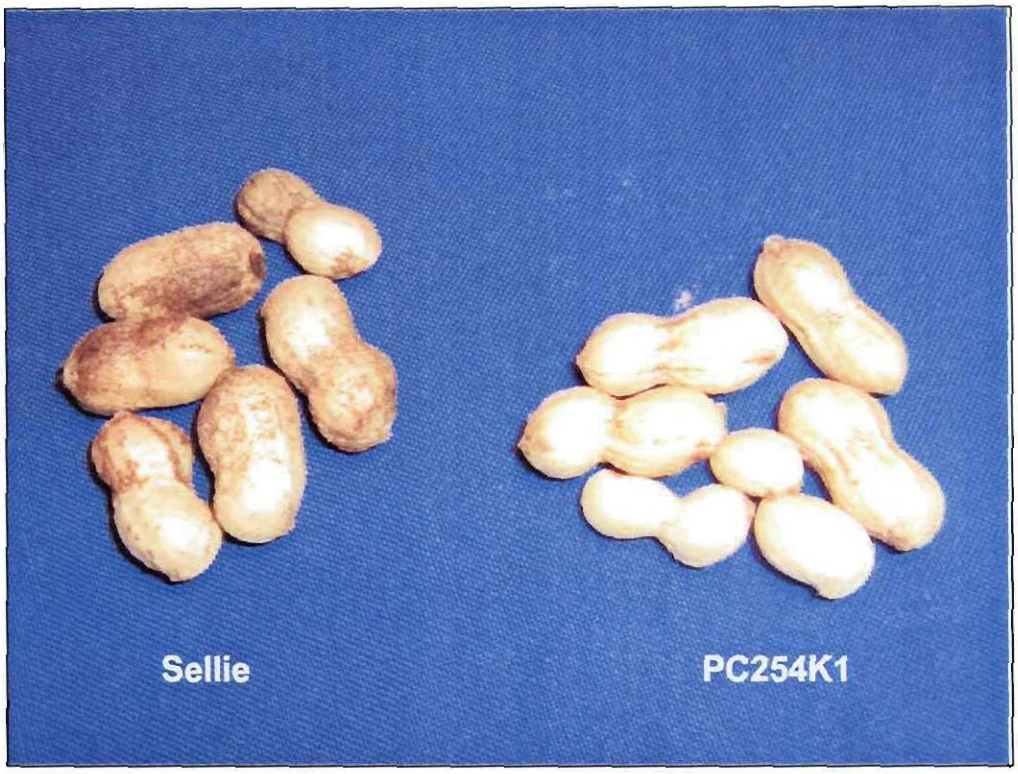

Figure 5.1. External symptoms on Sellie and PC254K1 pods at an inoculum level of 7 000 *Ditylenchus africanus* specimens 150 DAP in a greenhouse trial during 2008.

5.3.2 Histopathology

5.3.2.1 Pegs

The histopathology in longitudinally-sectioned pegs of Sellie at the various inoculum levels and different sampling times were similar in appearance to that of longitudinally-sectioned pegs from PC254K1 collected at similar inoculum levels and sampling times (Fig. 5.2 A & B). Each peg had a distinct exocarp with developing vascular bundles in the mesocarp. The inner layer of the mesocarp was differentiating into a fibrous mesocarp layer. The cells of the fibres were still thin-walled and unlignified (Fig. 5.2 A & B). In the central pith of the PC254K1 pegs a part of the fruit locule contained the ovule (Fig. 5.2 B). Within the ovule resided the embryo sac in which a groundnut embryo would be formed. No nematodes were observed in the peg tissue of either Sellie or PC254K1 from the nematode-free controls or any of the nematode inoculum levels at 90, 120 or 150 DAP.

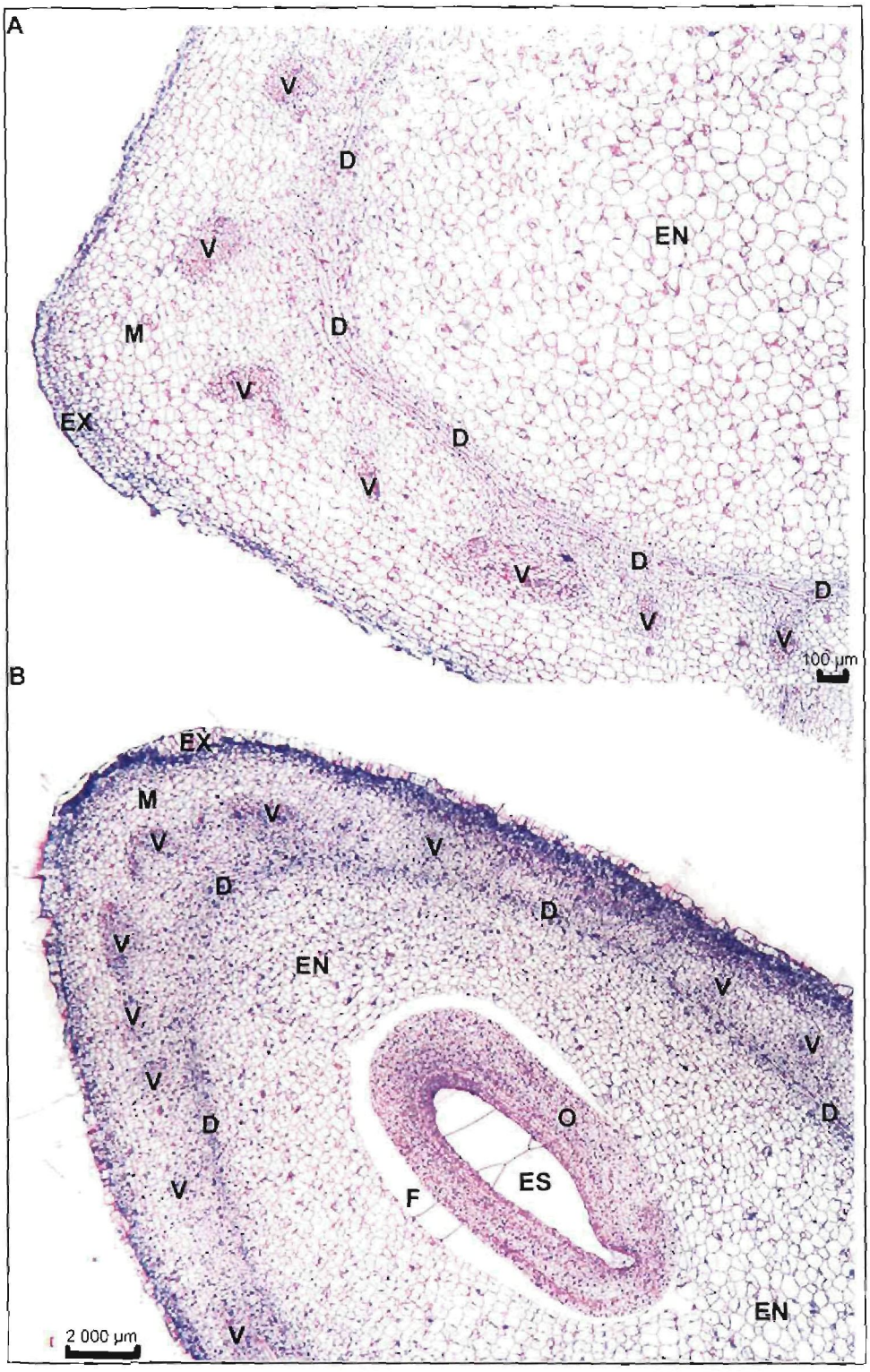

Figure 5.2. Light micrograph of a longitudinal section of a peg from A. Sellie and B. PC254K1 at an inoculum level of 7 000 *Ditylenchus africanus* specimens at 150 DAP from a histopathology study during 2008. A & B magnified 20 x. D = developing fibre layer, EN = endocarp, ES = embryo sac, EX = exocarp, F = fruit locule, M = mesocarp, O = ovule, V = vascular bundle.

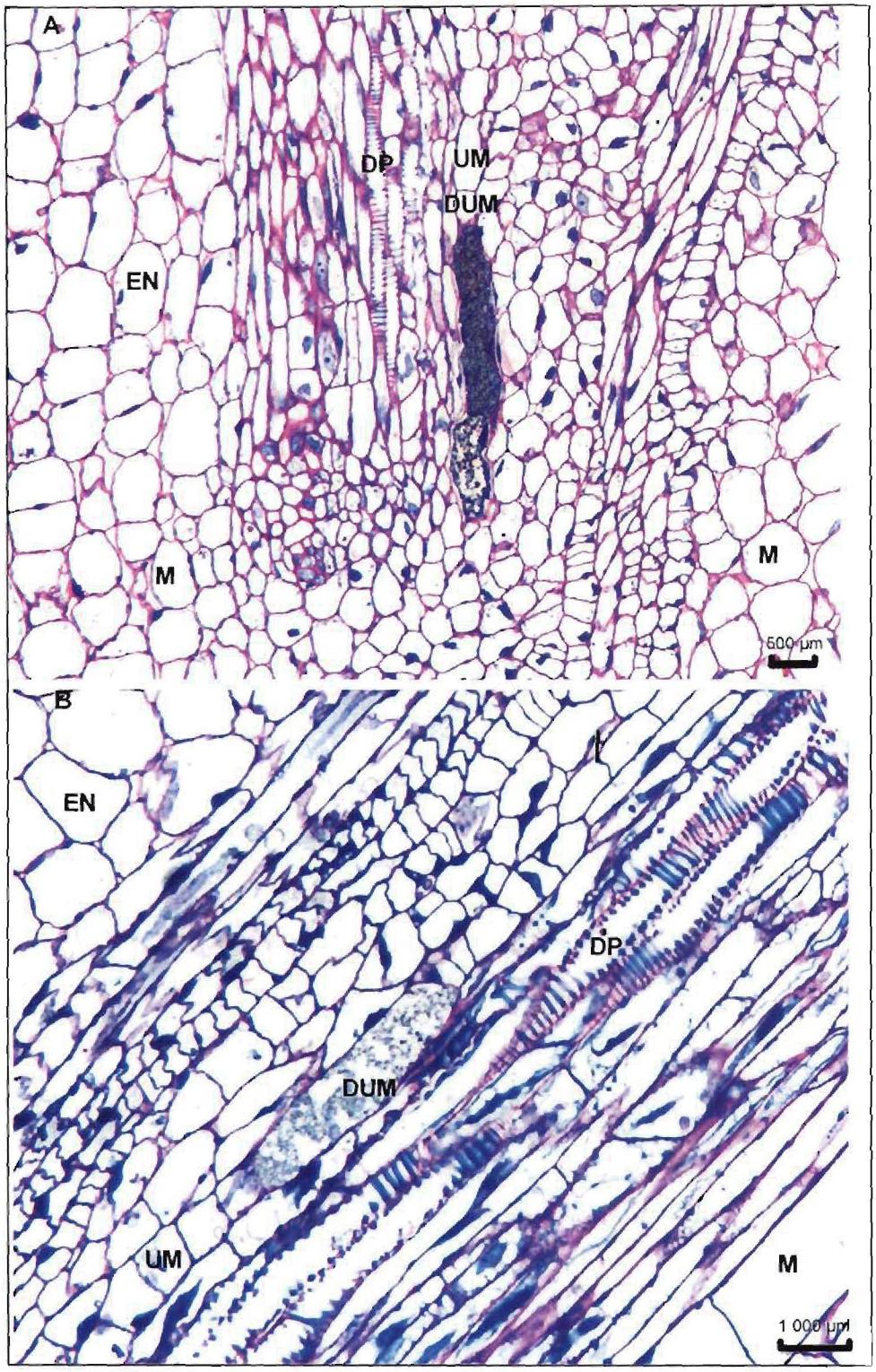

Figure 5.3. Light micrograph of a cross section through the vascular region of pegs from A. Sellie and B. PC254K1 at an inoculum level of 7 000 *Ditylenchus africanus* specimens at 150 DAP from a histopathology study during 2008. A & B magnified 100 x. DUM = damaged undifferentiated tracheary elements of the metaxylem, DP = differentiated tracheary elements of the protoxylem, EN = endocarp, M = mesocarp, UM = undifferentiated tracheary elements of the

metaxylem.

Cross sections of the vascular region of pegs of inoculated as well as uninoculated Sellie and PC254K1 at 90, 120 and 150 DAP were similar in appearance (Fig. 5.3 A & B). The vascular regions are situated in the parenchyma of the mesocarp adjacent to that of the endocarp. Each vascular region consists of a row of differentiated tracheary elements of the protoxylem that is thick-walled and has a ribbed appearance. Adjacent to the differentiated tracheary elements of the protoxylem the undifferentiated tracheary elements of the metaxylem consist of a long row of thin-walled cells. A number of cells along the undifferentiated tracheary elements of the metaxylem appear damaged and the cell walls between these cells are broken down. This damage along the undifferentiated tracheary elements of the metaxylem was also visible in Sellie and PC254K1 plants from the nematode-free control (not shown).

5.3.2.2 Pods

From 90 DAP *D. africanus* was observed in pod tissue of Sellie at all three different inoculum levels. At 150 DAP several nematodes were observed in Sellie pods at the 2 000, 5 000 and 7 000 inoculum levels. No nematodes were, however, observed in pods of Sellie from the nematode-free control at 90 and 150 DAP. Cross sections of mature pods of Sellie 150 DAP inoculated with ca 7 000 nematodes are presented in Figure 5.4 A & B. The funicle is surrounded by parenchyma cells of the endocarp (Fig. 5.4 A). Adjacent to the endocarp the cells of the fibrous layer of the mesocarp are thick-walled. Parenchyma cells of the mesocarp are located between the fibrous mesocarp and the corky layer of the exocarp. Nematodes were not present in the thick-walled cells of the vascular bundle of the funicle, in the thick walled cells of the fibrous mesocarp or in the cells of the exocarp. They were observed in the parenchyma cells of the mesocarp as well as in those of the endocarp (Fig. 5.4 A).

The largest proportion of the nematodes present in Sellie pods was observed around the vascular bundle of the funicle and in the parenchyma cells of the endocarp (Fig. 5.4 B). Walls of the malformed, elongated parenchyma cells around the nematodes that are located in the endocarp around the vascular bundle are broken down and cells started to aggregate. In advanced stages of parenchyma damage the cells completely collapse (Fig. 5.4 B). These cells were not malformed, aggregated or collapsed in pod tissue of uninoculated Sellie plants from the nematode-free control.

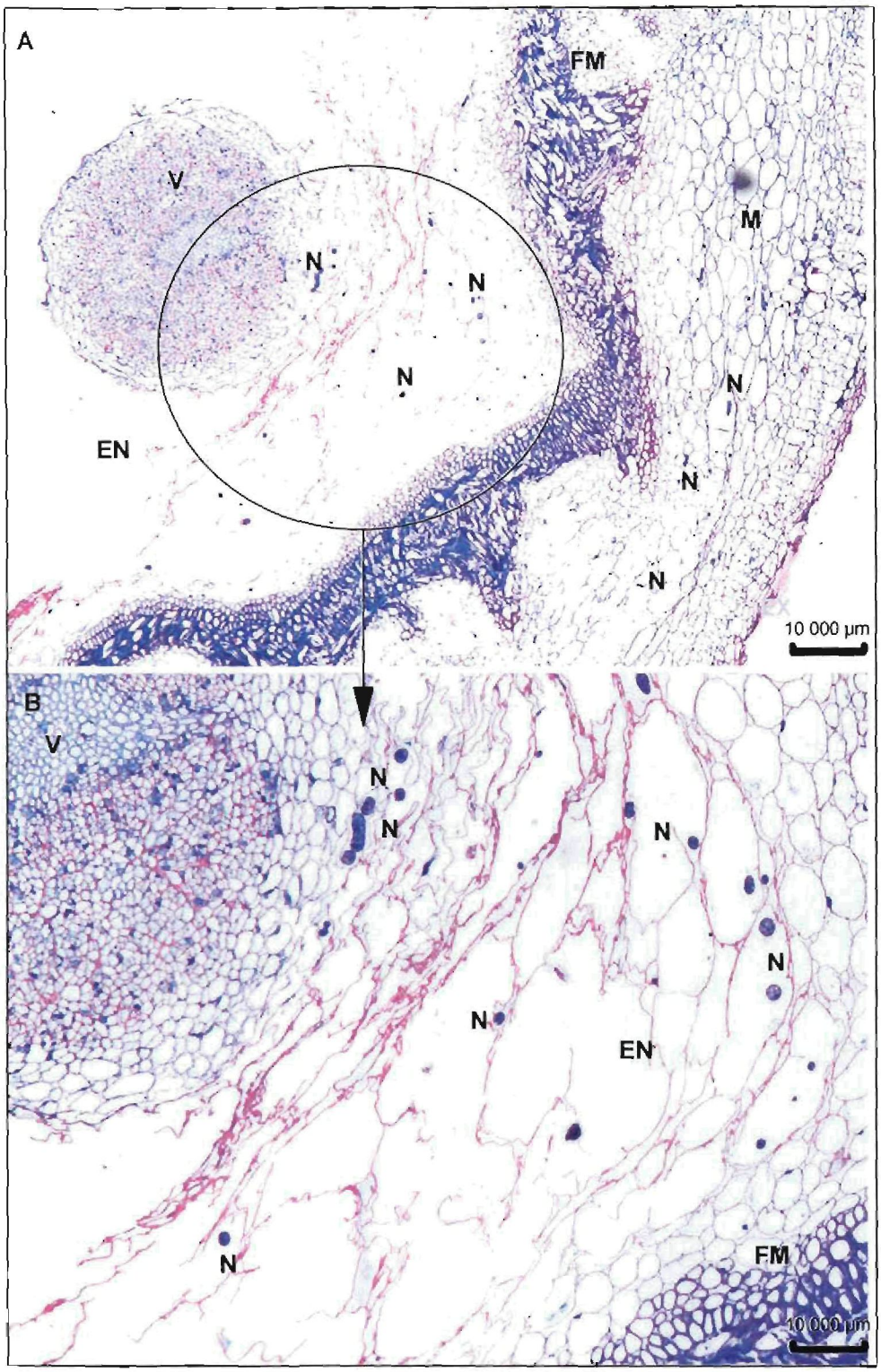

Figure 5.4. Light micrograph of a cross section through A. The vascular bundle of the funicle and surrounding endocarp and mesocarp and B. The vascular bundle of the funicle and surrounding endocarp of a mature, Sellie pod at an inoculum level of 7 000 *Ditylenchus africanus* specimens at 150 DAP from a histopathological study during 2008. A magnified 20 x and B 40 x. EN = endocarp, EX = exocarp, FM = fibrous mesocarp, M = mesocarp,

N = nematode, V = vascular bundle of the funicle.

Nematodes were observed in pods of PC254K1 for the 2 000, 5 000 and 7 000 inoculum levels at 90 DAP. No nematodes occurred in pods of PC254K1 and Sellie of the nematode-free control plants. Cross sections of the vascular region and surrounding mesocarp of a mature PC254K1 pod collected at 150 DAP from plants inoculated with ca 7 000 nematodes are presented in Figure 5.5 A & B. The vascular bundles are located in the mesocarp adjacent to the fibrous mesocarp, which consists of thick-walled cells. In contrast to the high numbers of nematodes observed in pod tissue of Sellie, few nematodes were seen in the pod tissue of PC254K1 (Fig. 5.5 A & B). *D. africanus* was not observed in the parenchyma cells of the endocarp, the thick-walled cells of the vascular bundles or in the thick-walled cells of the fibrous mesocarp of the latter genotype. They were observed only in cells in the mesocarp immediately adjacent to the thick-walled vascular bundles (Fig. 5.5 B).

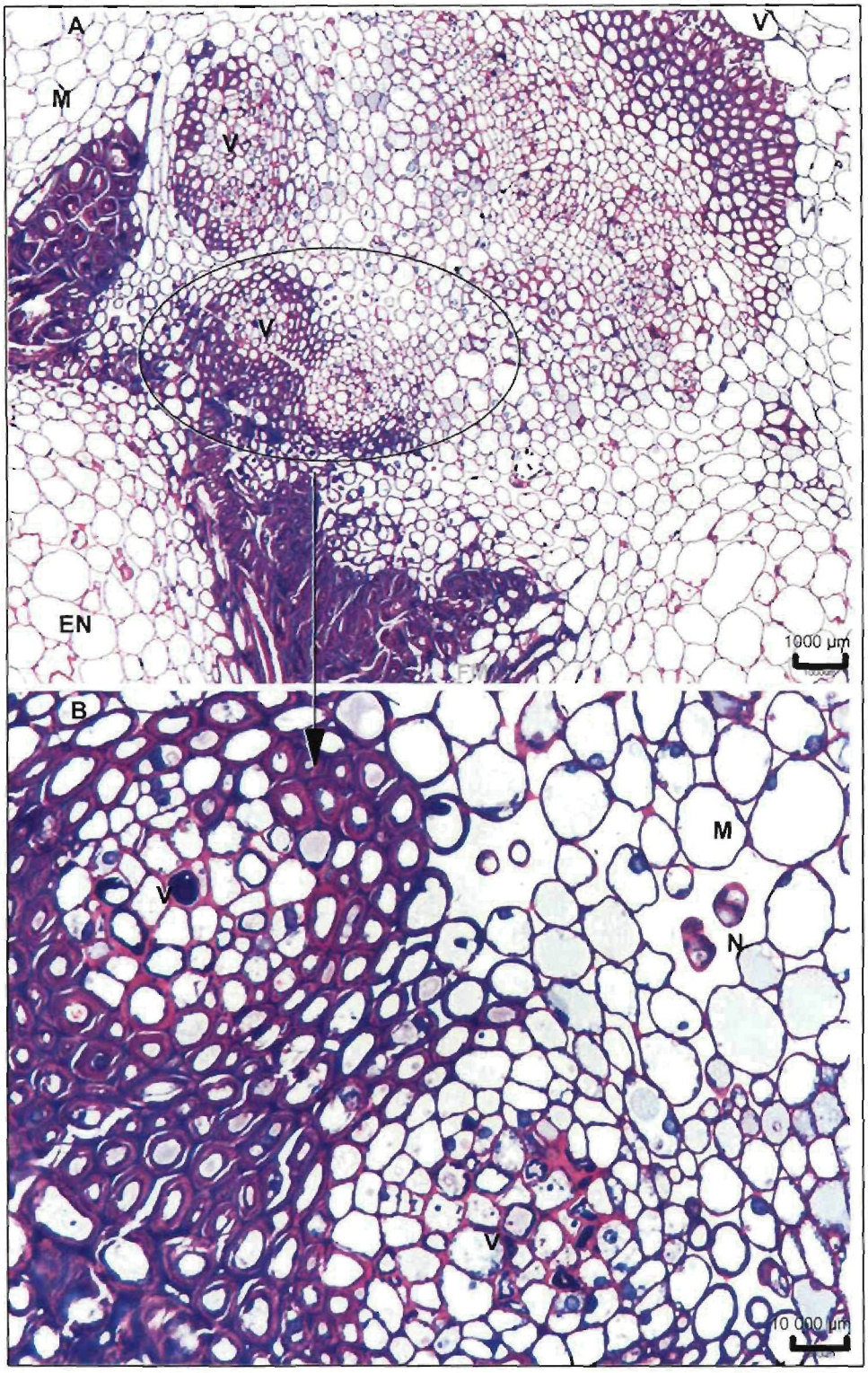

Figure 5.5. Light micrograph of a cross section through A. The vascular region and surrounding mesocarp and B. The vascular region of a mature PC254K1 pod at an inoculum level of 7 000 *Ditylenchus africanus* specimens at 150 DAP from a histopathology study during 2008. A magnified 20 x and B 40 x. EN = endocarp, FM = fibrous mesocarp, M = mesocarp, N = nematode, V = vascular bundles.

A region around the vascular bundles of a mature pod of PC254K1 at 150 DAP inoculated with ca 7 000 *D. africanus* specimens is presented in Fig. 5.6.

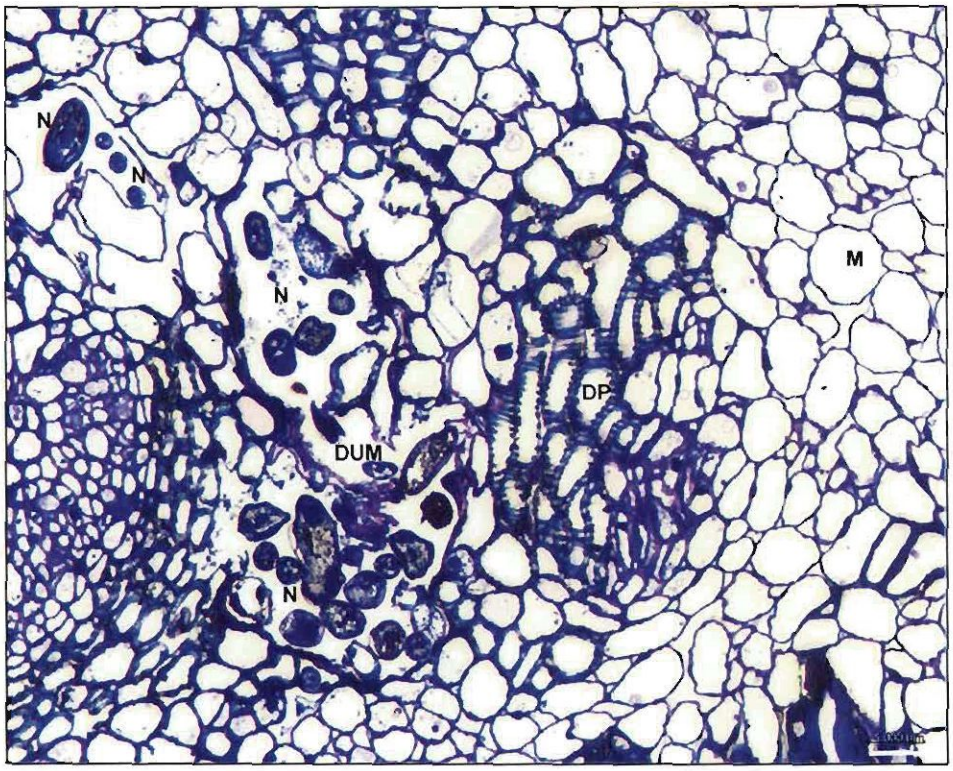

Figure 5.6. Light micrograph of a cross section through the cells of the vascular *region of a mature PC254K1 pod at an inoculum level of 7 000 Ditylenchus africanus* specimens at 150 DAP from a histopathology study during 2008. Magnified 100 x. DUM = damaged undifferentiated tracheary elements of the metaxylem, DP = differentiated tracheary elements of the protoxylem, M = *mesocarp*, N = *nematode*.

Differentiated tracheary elements of the protoxylem are thick-walled with a ribbed appearance similar to those in the peg tissue (Fig. 5.3 A & B). *D. africanus* was observed only in the area of the undifferentiated tracheary elements of the protoxylem. Parenchyma cells around the undifferentiated tracheary elements of the protoxylem were nematode-free, not malformed, aggregated or collapsed such as that of inoculated Sellie.

5.4 DISCUSSION

The brown discoloration on the pod surface and the necrotic tissue at the connection point of the peg to the pod observed on *D. africanus*-infected pods of Sellie during this study was similar to those observed on the same cultivar in previous studies (Jones & De Waele, 1988; De Waele *et al.*, 1989). Contrary to the situation with pods of Sellie, those of inoculated PC254K1 showed no external damage symptoms associated with *D. africanus*, confirming the resistance of this groundnut genotype to *D. africanus* even at high inoculum levels (also see Chapter 3).

Absence of *D. africanus* from peg tissue of inoculated Sellie of this study is in agreement with previous studies (Venter *et al.*, 1995). It is unclear what caused the damage to undifferentiated tracheary elements of the metaxylem in the pegs of inoculated Sellie as well as PC254K1 because similar damage was present in the same region in peg tissue of their uninoculated counterparts.

The damage caused by *D. africanus* in infected, mature Sellie pods at cellular level as well as the fact that individuals were present between the parenchyma cells of the mesocarp and endocarp but not beyond the mesocarp support observations made in previous studies (Jones & De Waele, 1988; De Waele *et al.*, 1989). Jones and De Waele (1990) and Venter *et al.* (1995) suggested that broken-down cell walls as observed in our study is a result of enzyme secretions of *D. africanus*. Seinhorst (1957) proposed that for proper development of *D. dipsaci* in host tissues, plant cells must be destroyed by dissolution of the middle lamella through enzymatic secretions by nematodes. In our study the macerated cells observed throughout the meso- and endocarp of infected Sellie pods indicated that this may also apply to *D. africanus*.

Lack of damage and the absence of *D. africanus* in pod tissue of PC254K1 confirmed the genotype's resistance and suggests that the mechanism of resistance may be inhibition of proper development and subsequent reproduction of *D. africanus*. The few nematodes found in the region of the undifferentiated tracheary elements of the metaxylem seemed not able to induce dissolving of the cell walls and the middle lamella of the neighbouring parenchyma cells (Seinhorst, 1957). As trachery elements mature, they lose their end walls through hydrolysis of the unlignified intercellular layer and their nuclei and cell contents, leaving hollow, dead cells that form vessels or tracheids (Fukuda, 1997). It appears as if hydrolysis of the walls by PC254K1 plants enabled a small number of *D. africanus* individuals to survive in

these tracheary elements. Therefore, although PC254K1 is not immune to *D. africanus* this genotype has the ability to prevent optimum development and reproduction of this nematode in its pods. This reaction of PC254K1 to *D. africanus* development and reproduction implies that antibiosis is most probably the mechanism of resistance involved.

CHAPTER 6

ORIGIN OF RESISTANCE TO *DITYLENCHUS AFRICANUS* IN PC254K1 AND IDENTIFICATION OF MOLECULAR MARKERS

6.1 INTRODUCTION

The groundnut breeding line PC254K1 has been identified during this study as a source with a high level of resistance to *D. africanus* and may have potential to be used in groundnut-breeding programmes (Chapters 3 to 5). Once a source of resistance has been identified a plant breeder needs to know the manner in which the trait is inherited (monogenic, oligogenic or polygenic) (Roberts, 1990) so that the most efficient breeding strategies could be selected for providing a desirable product that could enter the seed market by the shortest possible route (Boerma & Hussey, 1992).

Molecular markers could play an important role when improvement of the efficiency and duration of conventional plant breeding programmes is desired (Ortiz, 1998; Kasha, 1999). Markers that are closely linked to specific genes could facilitate verification of the presence of a specific trait as well as pyramiding of a number of desired genes into a single variety (Mienie *et al.*, 2005). The use of molecular markers is, therefore, widely accepted as valuable tools for the genetic improvement of various crops (Collard *et al.*, 2005). Church *et al.* (2001) identified the RFLP markers R2430E and S1018E that flanked a dominant resistance gene locus for root-knot nematode resistance in groundnut but since the resistance of PC254K1 to the groundnut pod nematode has only been identified during this study (Chapter 3), no information is available on markers associated with this specific trait.

Therefore, the objectives of this part of the study were to i) establish the mechanism of inheritance of the *D. africanus* resistance trait in PC254K1 and to ii) identify molecular markers linked to this trait.

6.2 MATERIALS AND METHODS

6.2.1 Origin of the resistant trait

6.2.1.1 First-generation crosses (F_1 seed)

This study was conducted in a greenhouse with a 18 °C to 30 °C temperature regime and a 13-h photoperiod. A total of eighteen 25 000-cm^3 plastic pots were filled with fumigated, sandy-loam soil (Chapter 2). The following nutrients were added to each pot: 36.5 g dolomitic lime, 22 g super phosphate (10.5 %), 14.5 g sodium chloride (KCl) and 9.4 g calcium nitrate ($CaNO_3$) mixed into the soil as described in Chapter 2.

Sellie and PC254K1 seed were obtained from nematode-free mircoplot nurseries and treated before planting with fungicide and nitrogen-fixing bacteria (Chapter 2). Plants used as male parents in the crosses were planted in two 1-week-consecutive batches for continuous availability of pollen for artificial pollination of female plants throughout the flowering phase. Each batch of males consisted of three pots planted with Sellie and three pots of PC254K1. The first batch was planted a week before planting of the females. A week later three pots each of Sellie and PC254K1 were planted to be used in the crosses as females and three pots each of Sellie and PC254K1 were planted to be used in the crosses as males. Female plants were kept apart from male plants. A plastic marker placed in each pot indicated the relevant groundnut genotype and whether it was used as female or male parent.

Crosses with Sellie as the male and PC254K1 as the female and *vice versa* (reciprocal crosses) were made as soon as the 18 female plants (nine Sellie and nine PC254K1 plants) started flowering approximately five weeks after planting. Young flowers situated on the first three nodes of the first three branches of female plants were used for the crosses because these parts carry approximately 74 % of the total number of pods at harvesting, which in turn produces approximately 92 % of the total kernel yield of a groundnut plant (Swanevelder, 1997). Flowers in these regions that were not used in crosses as well as those that developed elsewhere on the plants were removed on a daily basis to prevent undesirable self-pollinations. Crosses were made daily on flowers that have developed the previous day. Self-pollination of the developing flowers before artificial pollination was avoided by removing the anthers between 15:00 and 18:00 the day before maturation of each flower's own stigmata

(Knauft & Wynne, 1995). The lower lip of the calyx, wing and keel petals were removed with a sharply-pointed forceps to expose the anthers and stigmata. The anthers were then carefully removed without damaging the stigmata. Emasculated flowers were marked with pieces of coloured string, loosely tied around them so that they could be identified for artificial pollination the following day (Fig. 6.1).

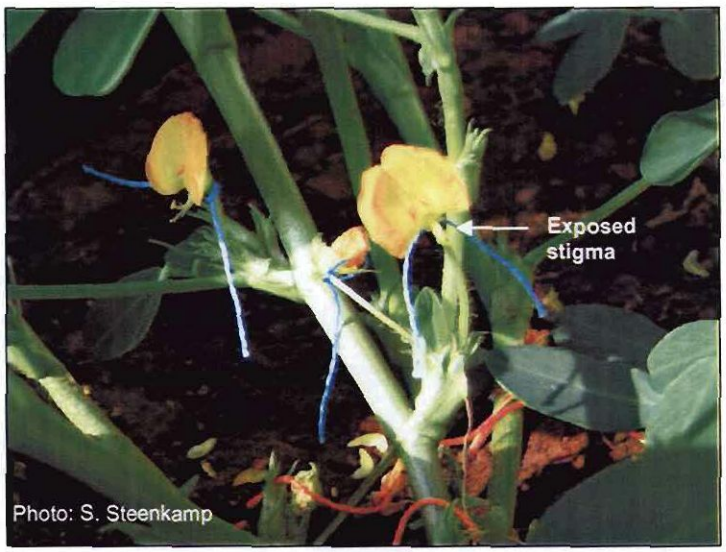

Figure 6.1. Emasculated flowers on a groundnut plant marked with coloured strings for artificial pollination.

Artificial pollination of flowers proceeded between 7:00 and 9:00 in the morning following emasculation because this is the period during the day when the stigmatic tissue is most receptive and pollen is most viable (Knauft & Wynne, 1995). Pollen was harvested from anther sacs of mature flowers on male parents just before pollination. A flower was removed from a male parent and ripe pollen was carefully squeezed from the anther sacs onto a forceps. The pollen was then carefully transferred onto a receptive stigma of an emasculated flower marked with a coloured string. The forceps used during artificial pollination was dipped in 70 % ethanol after each pollen transfer in order to prevent cross-pollination. To prevent dehydration of stigmata that were exposed during emasculation and of the pollen being transferred to the stigmata of female parents the latter flowers were covered with plastic bags after pollination. The plastic bags were each supported by 60 X 36-cm^2 wire frames (Fig. 6.2).

Figure 6.2. Female plants covered with a plastic bag supported by a wire frame.

Pegs that developed from 40 successfully pollinated flowers seven to 10 days after pollination were labelled to indicate the female and male parent. Mature pods that developed approximately five months after artificial pollination were removed from plants, placed in appropriately marked brown paper bags and were air-dried in a greenhouse for seven days. After this the pods were shelled to collect F_1 seeds. Twenty F_1 seeds were obtained from Sellie (♂) and PC254K1 (♀) and its reciprocal crosses, respectively.

6.2.1.2 Second-generation (F_2) seed

Seed of F_1 from the reciprocal Sellie and PC254K1 crosses was planted in a greenhouse specially adapted for breeding purposes (Fig. 6.3) during 2005-2006. The four 2.1 x 1.4 x 1.4-m^3 brick troughs are built over a drainage system to prevent waterlogging. Each trough was filled with EDB-fumigated, sandy-loam soil that was re-fumigated before planting of the trial (Chapter 2). Nutrient amendments consisting of 200 g dolomitic lime, 139 g super phosphate (10.5 % P), 42 g KCl and 40 g $CaNO_3$ per trough were incorporated into the top 30 cm of the soil of each of the troughs following procedures described in Chapter 2.

Figure 6.3. Groundnut breeding facility in a greenhouse.

Seed was treated with fungicide and inoculated with nitrogen-fixing bacteria (Chapter 2) before planting at an intra-row spacing of 10 cm and inter-row spacing of 45 cm (Chapter 2). In the first four adjacent rows the 20 F_1 seed from Sellie (♀) x PC254K1 (♂) crosses were planted and in the last four rows the 20 F_1 seed from PC254K1 (♀) x Sellie (♂) crosses. Plastic markers placed in front of the first row of each four-row block indicated the female and male parent. Plants were allowed to self-pollinate and were watered three times a week by hand. At harvesting the plants were lifted from the soil (Chapter 2) and the pods (Fig. 6.4) removed.

Figure 6.4. Mature pods produced by F_1 plants after crosses were made between Sellie and PC254K1.

The pods were placed in marked paper bags and air-dried in a greenhouse for seven days. After this they where shelled to obtain F_2 seed. Hundred and sixty-three seeds were obtained from the F_1 plants from Sellie (♀) x PC254K1 (♂) crosses and 159 seeds form the F_1 plants of PC254K1 (♀) x Sellie (♂).

6.2.1.3 Evaluation of the F_2 progeny for *D. africanus* resistance

During 2006-2007 the abovementioned F_2 progenies were evaluated for resistance to *D. africanus* in a microplot facility that consists of a row of 20 rectangular clay-brick troughs (Chapter 2). The troughs were filled with the same EDB fumigated, sandy-loam soil used in the pots in the greenhouse in this chapter (Chapter 2). Nutrient amendments per plot were 75 g dolomitic lime, 136 g super phosphate (10.5 % P), 40 g KCl and 41 g $CaNO_3$. The nutrients were incorporated into the top 30 cm of the soil as described in Chapter 2.

The trial was planted in a randomised complete block design. The four treatments consisted of the parents Sellie (susceptible) and PC254K1 (resistant), F_2 from Sellie (♀) x PC254K1 (♂) cross and F_2 from PC254K1 (♀) x Sellie (♂) cross. Four rows were planted in each trough at an intra-row spacing of 5 cm and an inter-row spacing of 45 cm (Chapter 2). Each row represented a treatment and contained 20 seeds. The F_2 seed was treated with fungicide and inoculated with *B. arachis* (Chapter 2). White plastic markers at the front end of each row indicated the treatment.

At planting each seed was inoculated with $\pm$ 3 000 *D. africanus* of various life stages. The nematodes for inoculation were extracted from *D. africanus* cultures and prepared for inoculation as described in Chapter 2. The trial was irrigated supplementary to rainfall (Chapter 2). Each plant was labelled after germination (see also 6.2.2.1).

Nematode assessments were done at harvesting on all F_2 plants. Nematode extractions were done from peg-, hull- and kernel-samples from each plant, counted under a research microscope and expressed as number of Pf per 15 g pods (Chapter 2).

6.2.2 Molecular marker identification

6.2.2.1 DNA extraction

Three weeks after germination the F_2 seedlings (6.2.1.3) from the PC254K1 (♀) x Sellie (♂) cross were each labelled and a unique number was allocated to each seedling. Eight leaves were sampled per plant and placed in appropriately marked 2-ml Eppendorf tubes. They were freeze-dried and stored at –20 °C until extraction of their DNA.

Total genomic DNA was isolated from the leaves using a modified hexadecyltrimethylammonium bromide (CTAB)-method (Saghai-Maroof *et al.*, 1984). Freeze-dried leaves of each plant were ground to a fine powder and sub-sampled to approximately 250 µl of fine leaf powder. Each sub-sample was then transferred to a 2-ml microfuge tube, topped with 700 µl CTAB buffer consisting of 100 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0), 20 mM ethylenediaminetetra acetic acid (EDTA) (pH 8.0), 1.4 M sodium chloride (NaCl), 2 % (w/v) CTAB and 0.2 % (v/v) β-mercaptho-ethanol and incubated at 65 °C for 1 h. After incubation the suspension (aqueous phase) was extracted with a 500 µl chloroform:isoamylalcohol solution (24:1 v/v). The aqueous and chloroform phases were separated by centrifugation of the suspension at 12 000 r.p.m. for 10 min. The chloroform phase was discarded and 0.02 $\mu g.\mu L^{-1}$ ribonuclease A (RNase A) was added to the aqueous phase contained in a microfuge tube and then incubated for 30 min at room temperature to digest RNA that could contaminate the DNA. DNA was centrifuged at 12 000 r.p.m. for 15 min and precipitated from the aqueous phase with ⅔ volume isopropanol. The precipitate collected in the microfuge tube was washed with 500 µl ice-cold, 70 % (v/v) ethanol and then centrifuged at 12 000 r.p.m. for 5 min. The pellet was air-dried for one hour and then resuspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). Absorbencies were measured at 260 nm and 280 nm to determine both DNA quantity and quality.

6.2.2.2 Amplified fragment length polymorphism (AFLP) analysis

AFLP analysis was conducted using a protocol modified after Vos *et al.* (1995). The primer pair combinations used to perform the analyses is presented in Table 6.1. *Mlu*I primers (MI) were screened in combination with *Mse*I-primers (M). The codes following MI or M refer to the three selective nucleotides at the 3'-end of the primer.

Table 6.1. *Mlu*I (MI) and *Mse*I (M) adaptor, primer+1 and primer+3 sequences used in amplified fragment length polymorphism (AFLP) analyses of genomic deoxyribonucleic acid (DNA) of PC254K1, Sellie and the F_2 generation resulting from a PC254K1 x Sellie cross.

Enzyme	Type	Sequence (5' – 3')
MI	Adaptor-F	CTCGTAGACTGCGTAAC
	Adaptor-R	CGCGGTTACGCAGTC
M	Adaptor-F	GACGATGAGTCCTGAG
	Adaptor-R	TACTCAGGACTCAT
MI	Primer+1	GACTGCGTAACCGCGT
	Primer+3	GACTGCGTAACCGCGTNN
		TNN = TTA, TCA, TGC, TTA, TCT, TGT, TTG
M	Primer+1	GATGAGTCCTGAGTAAN
		N = A, C, G, T
	Primer+3	GATGAGTCCTGAGTAANNN
		NNN = ACA, ACC, ACT, CAA, CAC, CAG, CAT, CCG, CGT, CTA, CTC, CTG,
		CTT, GAA, GAG, GGC, TAC, TTG

Primers and adaptors were synthesised by Applied Biosystems (Integrated DNA Techologies, UK). MI primers were fluorescently labelled using 6-carboxyfluorescein (6-FAM), NED™, VIC™ or PET™. Adapters were prepared as follows: Equimolar amounts of each relevant pair (Table 6.1) were combined in a 0.2-ml Eppendorf tube. The solution was heated for 10 min to 65 °C in a water bath and then allowed to cool down to room temperature to ligate the primers.

Genomic DNA (500 ng) was digested for 5 h at 37 °C using four units (U) *Mse*I in NEB Buffer2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM magnesium chloride ($MgCl_2$), 1 mM dithiotreitol (DTT), pH 7.9; New England Biolabs), followed by the addition of 50 mM NaCl and 5 U *Mlu*I before overnight incubation at 37 °C. Adapters were ligated to the restricted genomic DNA by addition of 50 pmol *Mse*I adapter, 5 pmol of *Mlu*I adapter and 1 U T4 DNA ligase. Ligation was allowed overnight at 16 °C.

Pre-amplification reactions were performed in 50-µl reaction mixtures consisting of 5 µl ligated DNA, 200 µM of each dNTP, 30 ng of each pre-amplification primer (MI- and M-primer+1) (Table 6.1), 1x Taq polymerase buffer, 2 mM $MgCl_2$ and 1 U Taq

DNA polymerase. Samples were amplified in a Thermo Electron Multiblock System using 30 cycles of 30 s at 94 °C, 1 minute at 56 °C and 1 min at 72 °C each. Quality and quantity of pre-amplification products were determined by electrophoresis in a 1.5 % (m/v) agarose gel and diluted 1:50 prior to selective amplification.

Selective amplification reactions were performed in a 20-µl reaction volume consisting of 5 µL of 50-fold diluted pre-amplification product, 30 ng MI+3 primer (Table 6.1), 30 ng M+3 primer, 2 mM $MgCl_2$, 100 $\mu g.mL^{-1}$ bovine serum albumin (BSA), Taq polymerase buffer and 0.02 $U.\mu L^{-1}$ Taq DNA polymerase. Samples were amplified for one cycle of 30 s at 94 °C, 30 s at 65 °C and 1 min at 72 °C. The annealing temperature was lowered 1 °C for each of nine cycles followed by 24 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C each.

Capillary electrophoresis was done on an ABI3130x DNA sequencer with 16 capillaries. Diluted (1:20) polymerase chain reaction (PCR) amplification products generated by three different primer combinations were mixed and loaded together with a GeneScan™-500 LIZ® size standard in HiDi formamide onto the ABI3130*xl*. DNA fragments resulting from the primer combinations were distinguished using the distinct emission spectra of the fluorescently-labelled MI primers, while the samples were separated in the same electrophoresis capillary. Analysis was performed using the GeneMapper Version 4.0 programme.

6.2.2.3 Bulk segregant analysis

This part of the study was done after nematode evaluations (6.2.1.3) because the percentage reproduction rate (RR) of the nematodes was used to distinguish between resistant (RR was less than 10 % of that of Sellie), segregating (RR was between 10 % and 100 % of that on Sellie) and susceptible (RR was 100 % or more than that of Sellie) F_2 plants (also see 6.3.1) to support this analysis. A total of 107 primer combinations were tested on the DNA from leaves of the individual plants of the two parents and bulks. The two bulks consisted of DNA extracted from the leaves collected from six F_2 plants that showed the highest resistance levels to *D. africanus* ($RR \leq 10$ % of that on Sellie) and that of leaves collected from six F_2 plants that showed susceptibility to *D. africanus* ($RR \geq 100$ % of that on Sellie). Primer pair combinations that generated informative polymorphisms between the parents and bulks (fragments present in resistant parent and bulk or in susceptible parent and

bulk) were tested on all of the individual F_2 plants (103) from the segregating population (RR were between 10 % and 100 % of that in Sellie).

PCR reactions on the 103 individual plants were done separately and then multiplexed by combining 1 µl of each of three different-coloured dyes for the same individual in 20 µl of 0.1xTE buffer. Diluted PCR multiplex reactions (1 µl) were added to 9 µl HiDi-formide/GS500LIZ contained in a 96-well microplate, denatured at 95 °C for 3 min and immediately cooled down by placing the microplate on ice. The plate was covered with an ABI3130 septum and briefly centrifuged at 12 000 r.p.m. before loaded onto the ABI3130*xl* for electrophoresis.

6.3 STATISTICAL ANALYSES

6.3.1 Origin of the resistant trait

RR of *D. africanus* was determined on each F_2 plant in the microplot trial to express the level of resistance (Timper *et al.*, 2003) and to separate resistant F_2 plants from susceptible ones (Church *et al.*, 2005). The equation used was RR = Pf (F_2) / Pf (Sellie) x 100. For preliminary hereditary evaluations the RR of the nematodes on the F_2 plants was combined in resistant (RR $\leq$ 10 % of that on Sellie) (Abdel-Momen *et al.*, 1998; Hussey & Janssen, 2002; Timper *et al.*, 2003), segregating (RR was between 10 % and 100 % than that on Sellie) and susceptible (RR $\geq$ 100 % than that of Sellie) to support bulk-segregant analyses (6.2.2.3). An incidence plot (Stat Graphics 5 Plus for Windows) was used to determine the distribution in the F_2 mapping population in terms of Pf in pods.

6.3.2 Marker identification

A binary matrix that reflected specific AFLP markers as present (1) or absent (0) was generated for each genotype using GeneMapper 4.0. Only markers that were reliable (association between the marker and *D. africanus* resistance trait was acceptable if the probability was $P \leq 0.05$) and repeatable were considered. The association between a marker and the *D. africanus* resistance trait was determined by doing a simple regression analysis for the F_2-mapping generation and dominant loci (genetic markers that occupy specific genomic positions within chromosomes) (AFLP analysis). Regression analyses of the data from the AFLP analysis were done using a linear model from StatGraphics Plus 5 for Windows. The genetic marker data

served as the independent variable and *D. africanus* numbers per 15 g pod (Pf) as the dependent variable. Association between the marker and *D. africanus* resistance trait was considered significant if the probability was $P \leq 0.05$. The R-squared statistic (R^2) was used as a measure of the magnitude of the association.

A Chi-square (Chi^2) analysis was performed to test distortion in segregation of the markers. Linkage analysis and drawing of the linkage map was done using MAPMAKER.EXP 3.0 (Lander *et al.*, 1987). MAPMAKER.QTL 1.1 (Paterson *et al.*, 1988) was used for the localisation of the resistance gene/quantitative trait loci (QTL) on the linkage map in a recessive model (Lincoln *et al.*, 1992) to scan linkage groups (LG) for the presence of QTL effects at a log-likelihood score (LOD) threshold of 3.0 in every 2.0 centi Morgan (cM) interval. A LG indicated the position and relative genetic distances in cM between the markers along the particular chromosome involved. The most important use of the constructed linkage map was to identify chromosomal locations of genes and QTLs (Collard *et al.*, 2005) associated with resistance to *D. africanus* and to indicate the position and relative genetic distances between markers along chromosomes (Collard *et al.*, 2005). Genes that are closely together or tightly linked will be transmitted together more frequently from parent to progeny than genes or markers that are located further apart (Collard *et al.*, 2005). The Haldane mapping function was used to assign markers to linkage groups. The position of the resistance gene on the map was determined by treating the trait as a marker and mapping it with other markers or by identifying the position of the resistance gene by using the scan command of MAPMAKER.QTL 1.1 as described by Herselman *et al.* (2004). Markers were assigned to linkage groups if the $LOD \leq 3.0$ and maximum recombination frequencies (θ) were ≤ 0.5.

6.4 RESULTS

6.4.1 Origin of the resistant trait

A total of 163 F_2 seeds resulted from the Sellie (♀) x PC254K1 (♂) cross in the greenhouse. *D. africanus* numbers (Pf) in the pods of the F_2 plants ranged from zero to 6 500 nematodes per plant. Forty-three F_2 plants showed resistance ($RR \leq 10$ %) and 37 F_2 were susceptible ($RR \geq 100$ %) (Table 6.2). The RR of 83 F_2 plants ranged between 11 and 99 % and could be classified as an intermediate group (segregating).

The F_2-plant population from the PC254K1 (♀) X Sellie (♂) cross consisted of 159 plants. Pf on the F_2 plants from the latter cross ranged from zero to 7 756 per plant. Fifty-one F_2 plants showed resistance (RR $\leq$ 10 %) and 35 F_2 plants were susceptible (RR $\geq$ 100 %) (Table 6.2). RR of the segregating F_2 plants (73) ranged between 11 % and 99 % and could be classified as an intermediate group.

Table 6.2. Percentage reproduction rate (RR) of *Ditylenchus africanus* on pods of F_2 plants from Sellie (♀) x PC254K1 (♂) cross and its reciprocal PC254K1(♀) x Sellie (♂) from a microplot trial during 2006-2007.

RR[1]	Sellie[2] (♀) x PC254K1[3] (♂) No of F_2 plants	PC254K1(♀) x Sellie (♂) No of F_2 plants
$\leq$ 10	43	51
$\leq$ 20	27	33
$\leq$ 30	24	8
$\leq$ 40	8	12
$\leq$ 50	9	9
$\leq$ 60	5	1
$\leq$ 70	4	3
$\leq$ 80	3	5
$\leq$ 90	3	2
$\geq$ 100	37	35

[1] RR = Pf (F_2) / Pf (Sellie) x 100

[2] Susceptible parent

[3] Resistant parent

When the F_2 plants were subjected to an incidence plot the distribution did not match the Mendelian laws for heredity (Klug & Cummings, 1994) for the Sellie (♀) x PC254K1 (♂) cross (Fig. 6.5) or its reciprocal (Figs 6.5 & 6.6). These plots showed a normal distribution in the F_2 plant population in terms of Pf.

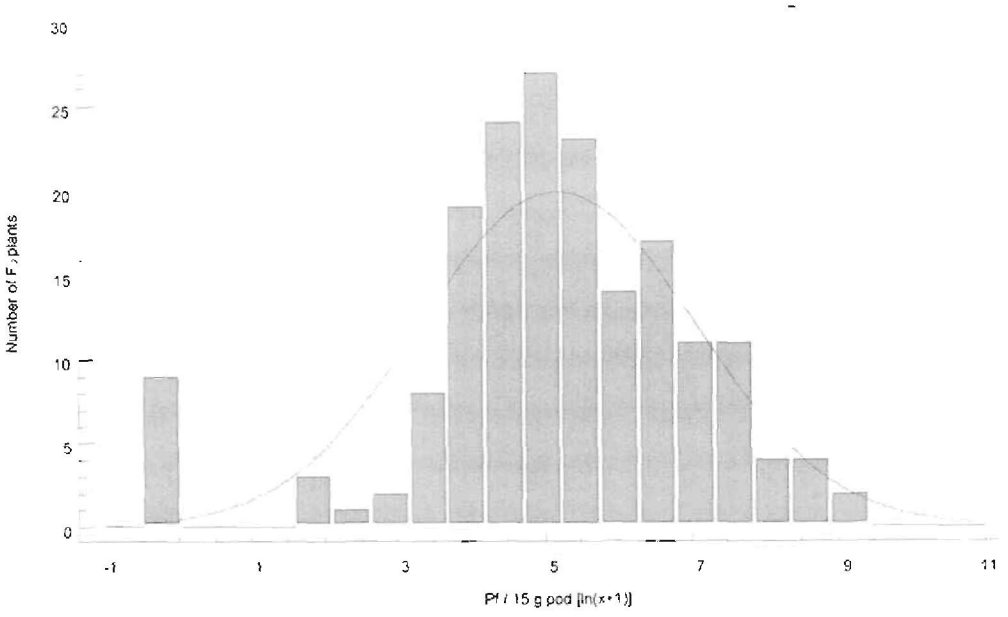

Figure 6.5. Distribution of F_2 plants from a cross between susceptible Sellie (♀) and resistant PC254K1 (♂) according to the mean number of *Ditylenchus africanus* ln(x+1) (Pf) in pods per plant at harvesting of a microplot trial during 2006-2007.

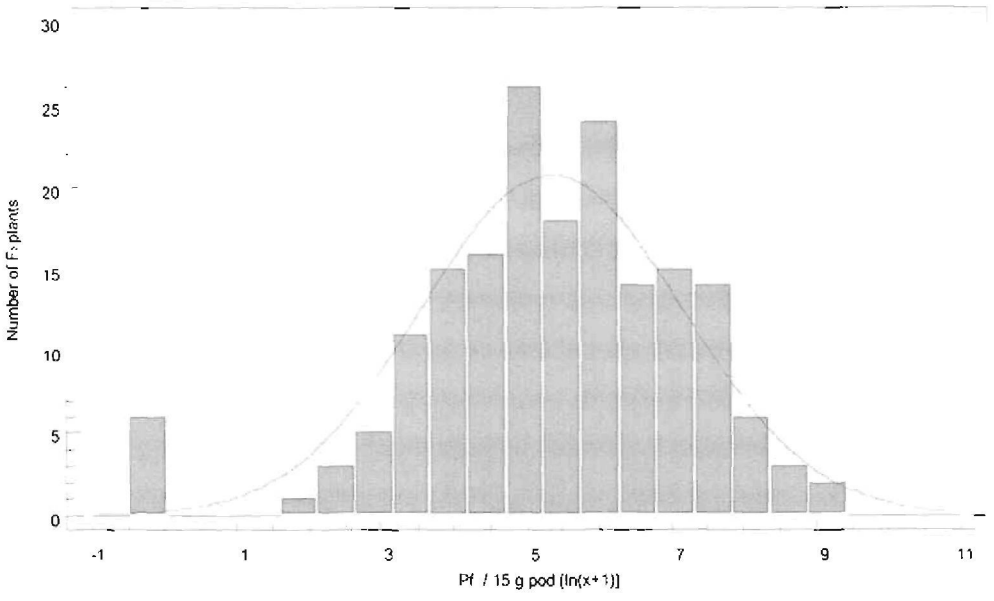

Figure 6.6. Distribution of F_2 plants from a cross between resistant PC254K1 (♂) and susceptible Sellie (♀) according to the mean number of *Ditylenchus africanus* ln(x+1) (Pf) in pods per plant at harvesting of a microplot trial during 2006-2007.

6.4.2 Molecular marker identification

6.4.2.1 AFLP analysis

Results obtained from the AFLP primers tested on the DNA of the parents and F_2 bulks are provided in Table 6.3.

Table 6.3. AFLP primers tested on the DNA of the parents PC254K1 (resistant) (♀) and Sellie (susceptible) (♂) and the F_2 progeny from PC254K1 x Sellie.

	*Mlu*I-*Mse*I reactions
Primer combinations tested	107
Total polymorphisms	430
Informative primers between parents and bulks	23
Average polymorphisms / primer combination	20.5
Maximum polymorphisms / primer combination	37
Minimum polymorphisms / primer combination	4

Hundred-and-seven primer combinations were tested during this study and a total of 430 polymorphisms were detected (Table 6.3). The polymorphisms included a minimum of four and a maximum of 37 polymorphisms per primer combination. Twenty-three of the 107 AFLP primers tested on the DNA of the parents and F_2 bulks were informative. These AFLP primers corresponded to fragments in the DNA from the resistant parent and resistant bulk of F_2 plants or to fragments in the DNA from the susceptible parent and susceptible bulk of F_2 plants. These informative AFLP primers were then further tested on the DNA of the 12 individual F_2 plants from the two bulks (6.2.2.3). Nine of the 23 informative AFLP primer combinations showed to be most useful and were tested on the entire population of 103 individual plants consisting of the 12 plants from the two bulks and 91 plants from the rest of the segregating population.

6.4.2.2 Linkage analysis

According to the Chi^2 test performed on the data 84 loci segregated into the expected 3 : 1 pattern ($P \leq 0.05$) (data not shown). These 84 loci were used for linkage mapping (Lander *et al.*, 1987). The nine primer combinations detected polymorphisms in the 91 plants of the segregating F_2 from the PC254K1 (♀) x Sellie

(♂) cross, which were used for the construction of a putative linkage map. Seven LGs were mapped (Fig. 6.7).

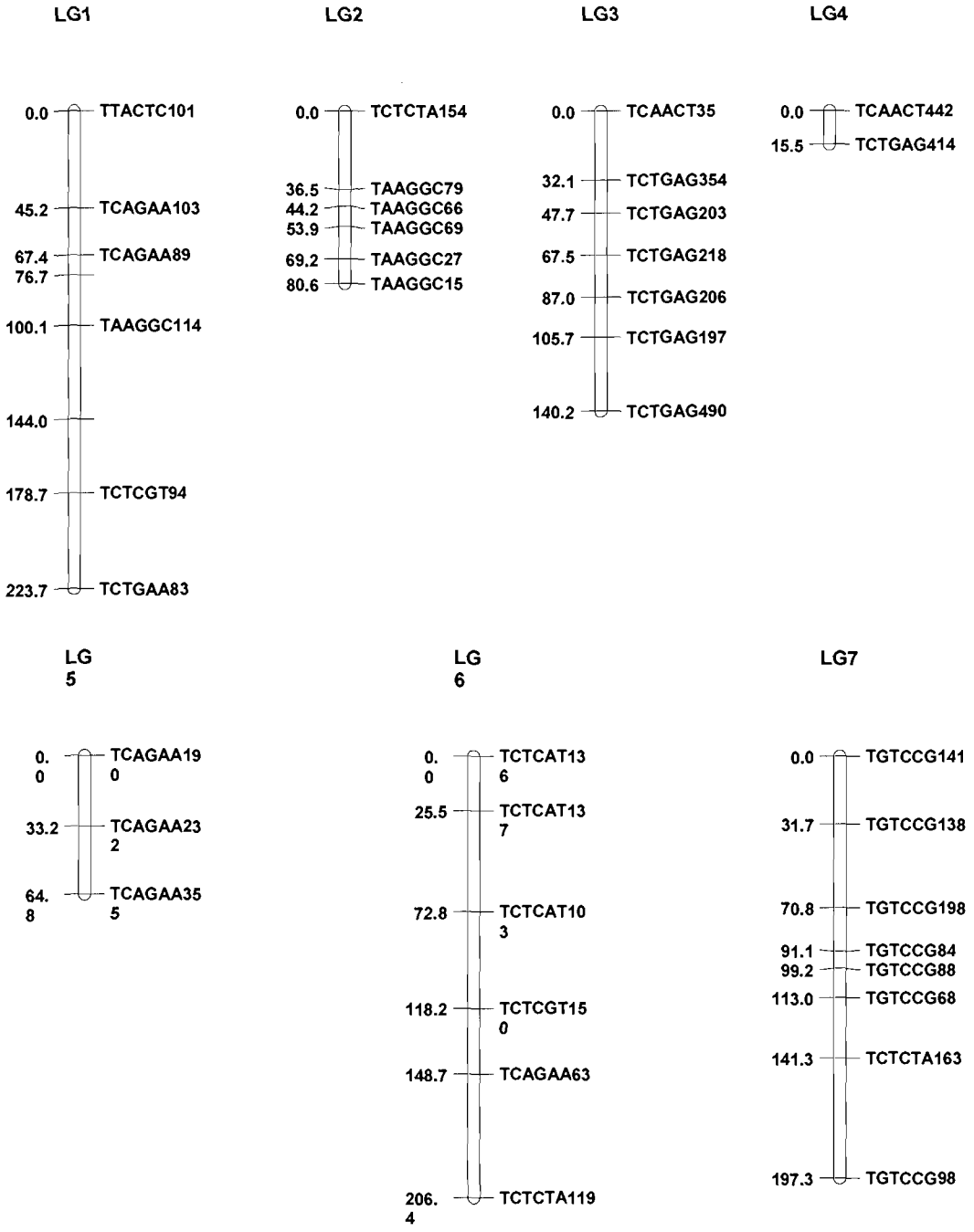

Figure 6.7. AFLP-based, genetic-linkage map developed for groundnut using a F_2 population resulting from a cross between the *Ditylenchus africanus* resistant PC254K1 and susceptible Sellie. Markers are indicated on the right of each bar and the distances between each marker are indicated in centi Morgan (cM) on the left of each bar.

Eight markers mapped to LG1, six to LG2, seven to LG3, two to LG4, three to LG5, six to LG6 and eight to LG7. Forty-four markers were linked with the resistance trait. LG1 covered a relative genetic distance of 223.7 cM, LG2 a distance of 80.6 cM, LG3 a distance of 140.2 cM, LG4 a distance of 15.5 cM, LG5 a distance of 64.8 cM, LG6 a distance of 206.4 cM and LG7 a distance of 197.3 cM. A total relative genetic distance of 928.5 cM was mapped on the seven linkage groups.

Results from the linear regression analysis on the DNA markers are shown in Table 6.4. Association between the marker and *D. africanus* resistance trait was considered significant if the probability was $P \leq 0.05$. Although TCTCGT102 (linkage group (LG) 1), TCTGAG490 (LG3), TCTGAG414 (LG4) and TCAGAA63 (LG6) showed a significant association with the resistance trait none of these markers showed to be closely linked to the trait since R^2 values were low.

Table 6.4. Linear regression analysis of DNA markers on the seven linkage groups (LG) on the AFLP-based genetic linkage map of groundnut. Loci were ordered using MAPMAKER.EXP and posistions for putative QTLs for *Ditylenchus africanus* resistance were determined using MAPMAKER.QTL.

Marker	LG	*D. africanus* / 15 g pod	
		P-value	R^2
TCTCGT102	1	0.037	4.9
TCTCGT94	1	0.112	2.9
TCAGAA103	1	0.193	2.0
TTACT101	1	0.221	1.6
TAAGGC114	1	0.423	0.8
TCAGAA89	1	0.475	0.6
TCAGAA83	1	0.624	0.2
TCAGAA86	1	0.789	0.1
TAAGGC79	2	0.402	0.8
TAAGGC69	2	0.455	0.7
TCTCTA154	2	0.496	0.6
TAAGGC66	2	0.528	0.5
TAAGGC115	2	0.778	0.1
TCTGAG490	3	0.034	7.0
TCTGAG354	3	0.074	5.0
TCTGAG203	3	0.111	4.0
TCTGAG206	3	0.460	0.9
TCAACT235	3	0.492	0.7
TCTGAG197	3	0.601	0.4
TCTGAG218	3	0.700	0.2
TCTGAG414	4	0.000	29.1
TCAACT442	4	0.213	2.4
TCAGAA232	5	0.147	2.4
TCAGAA190	5	0.944	0.0
TCAGAA355	5	0.953	0.0
TCAGAA63	6	0.001	11.5
TCTCTAT119	6	0.189	2.0
TCTCTAT137	6	0.325	1.0
TCTCTAT103	6	0.396	0.7
TCTCTAT136	6	0.424	0.6
TCTCGT150	6	0.468	0.6
TCTCCG138	7	0.153	2.1
TCTCCG198	7	0.310	1.1
TCTCTA163	7	0.532	0.5
TGTCCG84	7	0.519	0.4
TGTCCG88	7	0.511	0.4
TGTCCG141	7	0.531	0.4
TGTCCG68	7	0.601	0.3
TGTCCG98	7	0.606	0.3

6.4.2.3 QTL analysis

A QTL was detected on LG 4 between markers TCAACT442 and TCTGAG414 (Fig. 6.8). The log likelihood for this QTL was 6.97 and the variance for the *D. africanus* numbers explained was 58.2 % (data not shown). Two more putative QTLs were detected on LG 6, spanning the region between markers TCTCAT103 and TCTCTA119 (Fig. 6.8). The log likelihood of the one QTL between markers TCTCAT103 and TCAGAA63 on LG 6 were 13.03 and the percentage variance explained was 56.1 % (data not shown). The second QTL between markers TCAGAA63 and TCTCTA119 on LG 6 had a log likelihood of 12.12 and the variance explained for this QTL is 56.1 % (data not shown).

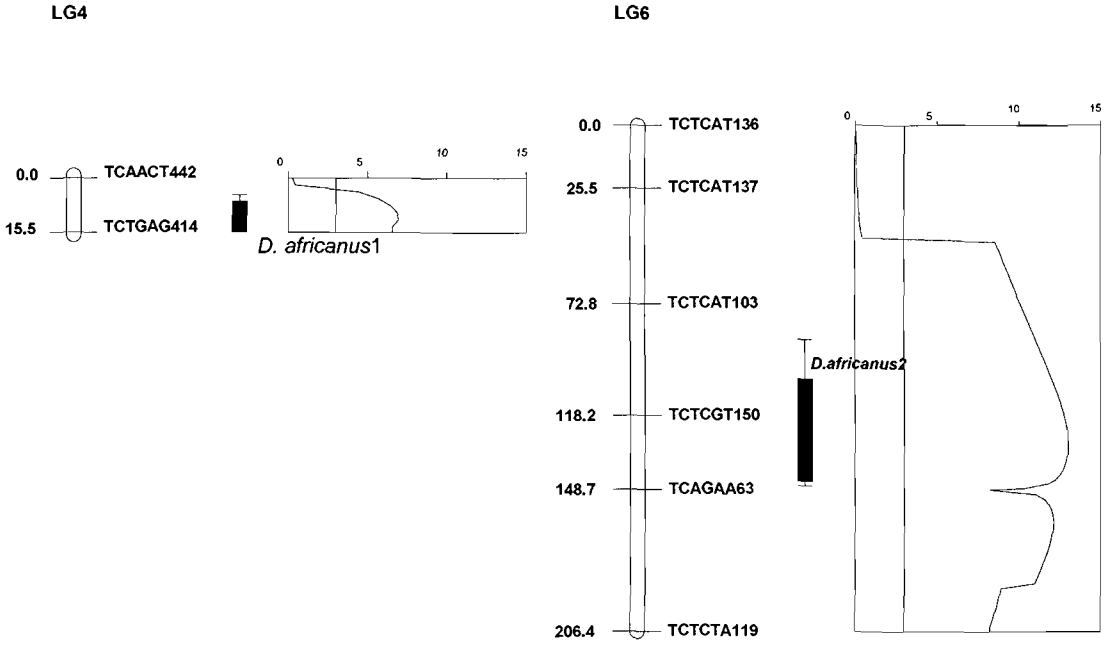

Figure 6.8. QTL-likelihood plot of markers for *Ditylenchus africanus* resistance on linkage groups (LG) 4 & 6. Markers are indicated to the right of each LG bar and the distances between each marker in centi Morgan (cM) to the left of the bar. Likelihood plots generated by MAPMAKER.QTL are indicated in blocks. The putative QTLs (*D. africanus* 1 & 2) are indicated by solid black bars.

6.5 DISCUSSION

Resistance or susceptibility of the parents and F_2 of the reciprocal Sellie and PC254K1 crosses was based on Pf of the nematodes in the pods at harvesting. This is where the largest proportion of a *D. africanus* population occurs at harvesting

(Basson *et al.*, 1990) and resistance can be determined with confidence only at pod maturity (De Waele *et al.*, 1990).

Although the Chi^2 segregation used in the linkage analysis were in the 3:1 pattern, the normal distribution of the F_2 plants in terms of nematode numbers on pods indicates that this trait was quantitatively (polygenic) inherited (Roberts, 2002) and does not comply with Mendelian laws for heredity (Strickberger, 1976; Klug & Cummings, 1994). The distribution of F_2 plants in terms of Pf in their pods also indicates that the resistance trait was polygenic (Roberts, 2002) because this trait demonstrated considerable variation and could not easily be categorised into resistant or susceptible classes (Klug & Cummings, 1994).

Although more polymorphisms were detected in groundnut during this study than those by Herselman *et al.* (2004) AFLP combined with bulk segregant analysis led to few informative fragments, with none that were closely linked with the *D. africanus* resistance trait. This is due to a lack of polymorphism for the markers between Sellie and PC254K1 due to the inherently narrow genetic base of this crop (Grieshammer & Wynne, 1990; Halward *et al.*, 1991; He & Prakash, 1997; Hopkins *et al.*, 1999; Subramanian *et al.*, 2000; Herselman, 2003). The lack of polymorphism for the markers between Sellie and PC254K1 complicated identification of the location of QTL's associated with the resistance trait during this study. Three QTL's have, nevertheless, been identified during this study on two separate linkage groups, which suggests strongly that, however strong, the resistance trait in PC254K1 is polygenic.

Introgression of the same level of resistance governed by polygenic genes into new cultivars may be difficult without the use of breeder-friendly markers, though. The position and proportion of linkage of the QTL marker, therefore, need to be validated by testing the reaction on an independent mapping groundnut population using CG7 (Chapter 3) as one of the parents. The AFLP markers can then either be used directly in a breeding programme or it can be converted to SCAR's (Sequence-Characterised Amplified Region).

CHAPTER 7

CONCLUSIONS

To achieve the goals set for this study a multi-disciplinary approach was taken. The combination of nematology, plant anatomy, plant breeding and molecular biology enabled us to provide a meaningful contribution to the local groundnut industry. On completion of this study the following objectives were reached:

i) The breeding lines PC254K1 and CG7 were identified as resistant to *Ditylenchus africanus*. The resistance expressed by these two genotypes is sustainable under field conditions.

ii) The resistance expressed by PC254K1 is effective even at high population densities. This genotype consistently produced yields with low UBS % at all Pi or Pf levels tested during this study, which supports the nematode data.

iii) The resistance expressed by PC254K1 is not transferred to leaf callus tissue of this genotype, confirming there is no short-cut for screening for resistance to *D. africanus*.

iv) The reproduction and damage potential of *D. africanus* populations from different localities in the groundnut-production areas of South Africa is similar. PC254K1 expressed resistance against all the *D. africanus* populations. This genotype sustained low nematode numbers and consistently produced yields with low UBS %.

v) The mechanism of resistance expressed by PC254K1 seems to be the inhibition of proper development and reproduction of this nematode.

vi) Origin of the resistance trait in PC254K1 seems to be polygenic.

vii) Eighty-four markers were mapped but none were closely linked to the resistance trait in PC254K1. More success may be achieved in future studies using the genotype CG7 from which PC254K1 was developed.

This study was pioneering because no worthwhile resistant groundnut sources were identified since the discovery of *D. africanus* during 1987. PC254K1 and CG7 were the first groundnut genotypes identified with sustainable resistance to *D. africanus* under microplot as well as field conditions. In the presence of this nematode these two genotypes consistently maintained low nematode numbers and produced yields with low UBS %. PC254K1 furthermore sustained resistance even under high

nematode population pressure. The strong characteristic of PC254K1 in terms of *D. africanus* population suppression is of particular significance because the Pi of *D. africanus* in a field is irrelevant when current cultivars are grown since this nematode is able to build up to damaging levels during a single growing season even from a relatively small Pi. PC254K1 could, therefore, be used a major source of resistance to *D. africanus* in a groundnut-breeding programme for developing resistant cultivars. Although PC287K5 also maintained low nematode numbers in some trials, its level of resistance does not seem to be as strong as that of PC254K1 or CG7 and seems not to be as sustainable under field conditions. However, this line could still play an important role in the groundnut industry particularly under conditions of lower *D. africanus* infestations. Previous studies have shown that even cultivars such as Kwarts that show tolerance to this nematode could play a significant role under certain conditions.

Development of a *D. africanus*-resistant cultivar will benefit to the groundnut industry because input costs could be reduced, which in turn would increase a producer's profit margin especially under dry-land conditions.

The ultimate challenge for any breeding-programme is to release new cultivars through the shortest possible route. One method to theoretically reduce the period of evaluations of groundnut genotypes against *D. africanus* is the use of callus tissue cultured from the leaves of genotypes to be screened. Unfortunately it was confirmed that the resistance expressed by PC254K1 in microplot and field trials was not transferred to callus tissue. The exercise did, however, confirm the suitability of groundnut leave-callus tissue cultures for propagation of *D. africanus*.

The reproduction and damage potential of *D. africanus* populations from different geographically-isolated localities in the groundnut-production areas of South Africa was similar. Resistance of PC254K1 to all of the populations tested was confirmed. These results indicate that the resistant trait of a *D. africanus* cultivar developed from PC254K1 should be sustainable over the whole groundnut production area of South Africa.

The absence of *D. africanus* from pod tissue of PC254K1 confirmed the genotype's resistance and suggested that the mechanism of resistance may be the inhibition of proper development of this nematode. The few *D. africanus* individuals observed near vascular bundles were not able to affect dissolving of the cell walls, which is

necessary for proper development and reproduction of the nematode. *D. africanus* is, therefore, not able to develop and build up to damaging population levels on PC254K1. The presence of low nematode numbers also means, however, that PC254K1 is not immune to this nematode. When PC254K1 would be rotated with susceptible genotypes these low nematode population levels may reach damaging proportions on the susceptible genotype over time. Additional control measures would still need to be applied.

Information on the mechanism of resistance is important to groundnut breeders. Combining the resistance in PC254K1 with that of a resistant genotype from a different source or with other desirable characteristics could produce a cultivar of superior preference in the groundnut industry.

The resistance trait in PC254K1 is seemingly governed by a number of genes. On the one hand this implies that resistance of PC254K1 will be more durable under constant selection pressure of *D. africanus* populations. However, although markers associated with the resistance trait were mapped, they were not closely linked to the resistance trait because of the lack of polymorphism between the parents. Three putative QTL's were identified but markers associated with the resistance trait need to be refined and developed into breeder-friendly markers that could be used in marker-assisted selection. These results cast doubt on the inheritance of this resistance, at least at the level expressed throughout the study.

CG7 is one of the parents of PC254K1 and was tested only in one part of the study but there are strong indications that this genotype may have superior levels of resistance to *D. africanus* that may even surpass that of PC254K1. Identification of markers closely associated with the resistance trait might, therefore, be more successful using CG7 in stead of PC254K1. The quest for useful markers should be continued not only to expedite introgression of this valuable source of *D. africanus* resistance but also to ascertain that the level of resistance is sustained in commercial cultivars resulting from a resistance-breeding programme.

CHAPTER 8

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