Differential responses of resistant and susceptible groundnut genotypes at cellular level to *Ditylenchus africanus*

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Summary- *Ditylenchus africanus* causes cellular breakdown in pod tissue of susceptible groundnut cultivars. The histopathology of this nematode on a resistant genotype was studied using light microscopy and compared with the histopathology of *D. africanus* on a susceptible genotype. Plants of breeding line PC254Kl and cv. Sellie were propagated in a glasshouse, inoculated with *D. africanus* at inoculum levels of 2000, 5000 and 7000 nematodes per plant and the pods were collected at 90, 120 and 150 days after planting. In contrast to the susceptible genotype, only a small number of nematodes were observed in restricted areas of the pod tissue of the resistant genotype. Furthermore, the resistant genotype showed neither external symptoms nor cellular breakdown in reaction to *D. africanus.* According to results of this study, the mechanism of resistance involved may be the inhibition of proper development, migration and reproduction of this nematode, thus preventing it building up to damaging population levels.

Keywords- *Arachis hypogaea,* histopathology, host response, mechanism of resistance, nematode damage, resistance.

Ditylenchus africanus is currently considered to be economically one of the most important plant parasites that limit groundnut production in South Africa (Jones & De Waele, 1988; Venter *et al.,* 1991; Swanevelder, 1997). This nematode causes severe losses in groundnut crops and hence income for producers (Me Donald *et al.,* 2005). Symptoms of *D. africanus* infections have a negative effect on the percentage of unsound, blemished and soiled (UBS%) kernels (Venter *et al.,* 1991; Vander Merwe & Joubert, 1992; Me Donald *et al.,* 2005) and are highly correlated with the number of nematodes found in the testa of infected groundnut seed (Venter *et al.,* 1991; Me Donald *et al.,* 2005).

The histopathology of *D. africanus* has previously been studied on groundnut cv. Sellie, which is highly susceptible to this nematode (Jones & De Waele, 1988; Venter *et al.,* 1995). The nematode causes extensive tissue damage to hulls and seeds that manifests as malformations, wall breakage and collapse of cells. These effects were ascribed to enzymatic secretions from the nematodes (Venter *et al.,* 1995). Until recently, there has been no groundnut cultivar available with resistance against *D. africanus* (Easson *et al.,* 1990; Vander Merwe & Joubert, 1992). Therefore, no information is available on the histopathology of this nematode on resistant groundnut genotypes. The objective of this study was to compare the histopathology of *D. africanus* on a resistant line, PC254K1, with that on cv. Sellie in order to determine the mechanism of resistance involved in the former groundnut genotype.

Materials and methods

For this study PC254K1 and cv. Sellie plants were propagated in a glasshouse under conditions of optimum growth for the genotypes, allowing uninhibited development and feeding of D. *africanus.* The microscope slides prepared from the PC254K1 and cv. Sellie material were studied using a light microscope.

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EXPERIMENTAL DESIGN

Twenty-eight plastic pots were filled with 4000 cm^3 EDB-fumigated (1.162 g a.s. $2m^{-2}$), sandy-loam Hutton soil consisting of 93.6% sand, 3.9% clay, 1.9% silt and 0.6% organic material. Nutrients (5.80 g dolomitic lime, 3.45 g superphosphate (10.5% P), 2.25 g sodium chloride (KCl) and 1.60 g calcium nitrate ($CaN0_3$)) were added per pot based on a soil analysis as well as nutrient guidelines for grounduut production (Swanevelder, 1997) and mixed into the soil. Pots were watered 1 day before planting to enhance germination of the seed (Swanevelder, 1997).

Seed from both groundnut genotypes were obtained from the germplasm bank of the Groundnut Breeding Unit of the ARC-GCI. Prior to planting, 5 g of the seed of each genotype were soaked in water for 24 h (Bolton *et al.*, 1990) to confirm that the seed was *D. africanus*-free. Seed was treated before planting with standard fungicide (120 g Thiolin® 50 (kg seed)$^{-1}$; Thiolin, Almond Agro Chemicals (Edms) Bpk, Van Riebeeck Park, South Africa) and nitrogen-fixing bacteria (250 g Soygro® 50 (kg seed)$^{-1}$; Soygro (Edms) Bpk, Potchefstroom, South Africa). Treated seeds were planted directly afterwards to a depth of 5 em in the soil relevant to the specific method of groundnut production (Swanevelder, 1997). One seed was planted per pot.

The trial was conducted at 18-27oC and a 13 h photoperiod. Six pots each planted with cv. Sellie and PC254K1 were inoculated at planting with zero (nematode-free), 2000, 5000 or 7000 *D. africanus* of various life stages. The nematodes used for inoculation were extracted from *in vitro* D. *africanus* groundnut callus tissue cultures maintained in a growth cabinet at 26oC (Van der Walt & DeWaele, 1989). *Ditylenchus africanus* groundnut callus tissue cultures were initiated using nematodes originally extracted from pods (Bolton *et al.*, 1990) collected from *D. africanus-infested* fields in the Vaalharts irrigation scheme (Northern Cape Province). The pots were rotated clockwise every fortuight to eliminate the effect of temperature or light gradients in the glasshouse and the plants were watered three times a week.

Pegs not yet developed into mature pods as well as mature pods were collected at 90, 120 and 150 (at harvesting) days after planting (DAP). The pegs and pods of these plants were removed and mixed. Ten randomly-picked pegs and pods were then collected from each replicate. Pegs and pods used for the study were washed free of soil under a gentle stream of running tap water and fixed in 4% paraformaldehyde. In addition, pods collected at 150 DAP were photographed to compare external D. *africanus* damage symptoms between the two genotypes before fixing the material in paraformaldehyde as described above. This was done only at 150 DAP because that is the period when D. *africanus* populations peak in the pods (Easson *et al.*, 1990). External damage caused by these nematodes is, therefore, supposed to be optimal at 150 DAP.

All plant material was stored at room temperature for a maximum of 3 days after being fixed in paraformaldehyde before preparation for light microscope studies commenced.

PREPARATION OF PLANT TISSUE FOR LIGHT MICROSCOPE STUDIES

The plant material was prepared at room temperature under a fume hood. Pieces of peg and pod tissue were cut in approximately 2 mm sections and further fixed in Todd's fixative (pH 7.5) for 2 h (Todd, 1986). After 2 h the fixative was replaced with 0.05 M sodium cacodylate buffer in which the tissue samples were washed three times for 15 min each. The material was then post-fixed in 1% osmium tetroxide for 1 hand washed in three changes of cacodylate buffer for 15 min each. The washed material was dehydrated in an ethanol series of 50, 70, 90 and 100% for 15 min each. The material was then impregnated and embedded in LR® white resin (O'Brien & Me Culley, 1981). Sections (1 /LID, longitudinal and cross-sections) of the plant material were cut with glass knives using a rotary microtome, mounted on microscope slides and air-dried. The sections were stained with 0.5% aqueous 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 was allowed to dry for 3 min before it was mounted in eutellan and covered with a cover slip. Light micrographs were digitally recorded from selected slides using a Nikon E400 camera with motic image analysis software.

Results

EXTERNAL DAMAGE SYMPTOMS ON MATURE PODS COLLECTED AT 150 DAP

At 150 DAP symptoms of damage were visible on pods of cv. Sellie inoculated with *D. africanus* and symptoms were most severe at the highest inoculum level (Fig. 1). Upon inspection of Sellie pods the tissue at the connection

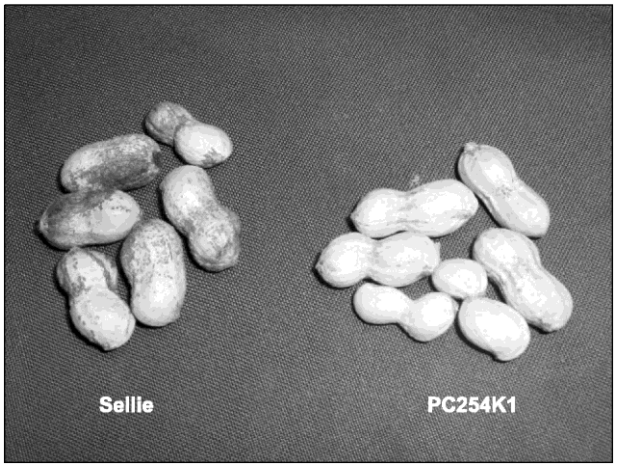

Fig. 1. *External symptoms on cv. Sellie and PC254K1 inoculated with 7000* Ditylenchus africanus *at 150 days after planting.*

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 cv. Sellie this dark discoloration extended over 75% or more of the pod surface. At 150 DAP pods of PC254K1 inoculated with 7000 *D. africanus* were not visually discoloured at the peg-pod connection point or over the pod surface (Fig. 1). No discoloration at the peg-pod connection or the pod surface was visible on Sellie or PC254K1 from the nematode-free control at 150 DAP.

HISTOPATHOLOGY

Pegs

The histopathology in longitudinally-sectioned pegs of cv. 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. 2). 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 in immature pods (Fig. 2). In the central pith of the PC254K1 pegs a part of the fruit locule contained the ovule (Fig. 2B). Within the ovule resided the embryo sac in which a groundnut embryo would form. No nematodes were observed in the peg tissue of either cv. Sellie or PC254K1 for both the nematode-free controls or the pegs of infested plants at 90, 120 or 150 DAP.

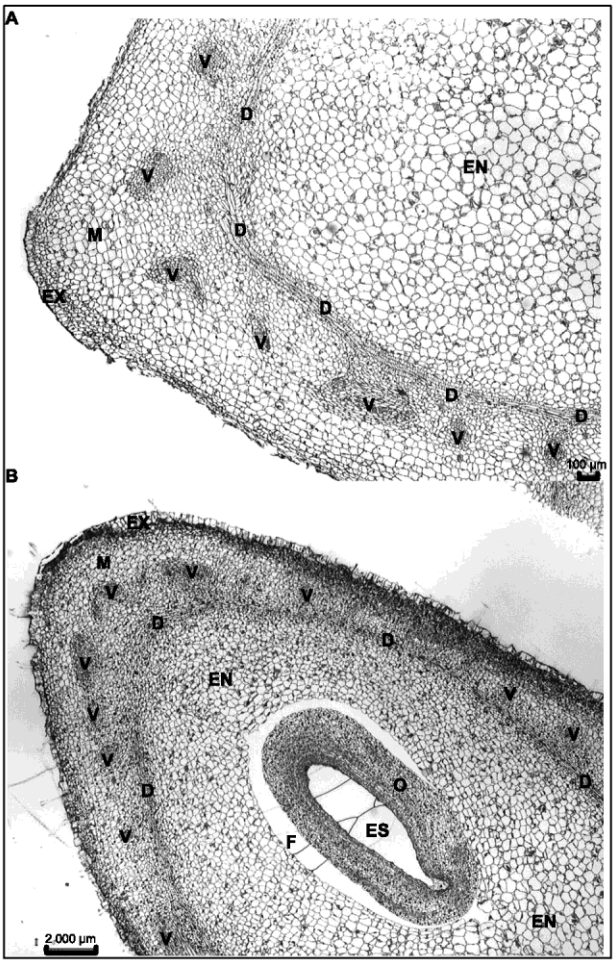

Fig. 2. *Light micrograph of a longitudinal section of a peg from cv. Sellie (A) and PC254K1 (B) inoculated with 7000* Ditylenchus africanus *at 150 days after planting. (Scale bars: A = 100 tJm; B = 2000 f1111· D = developing fibre layer, EN = endocarp, ES = embryo sac, EX = exocarp, F = fruit locule, M = mesocarp, O = ovule, V = vascular bundle.)*

Cross-sections of the vascular region of pegs of inoculated as well as non-inoculated cv. Sellie and PC254K1 at 90, 120 and 150 DAP were similar in appearance (Fig. 3). The vascular regions were 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 consists of a long row of thin-walled cells. A number of cells along the undifferentiated tracheary elements of the metaxylem appeared damaged and the cell walls between these cells were bro-

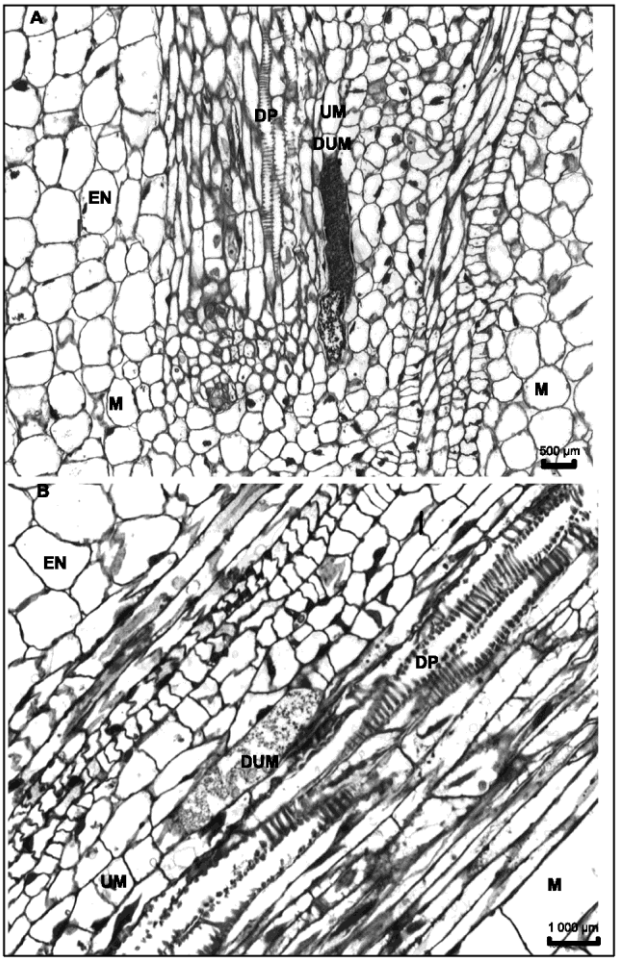

Fig. 3. *Light micrograph of a cross-section through the vascular region of pegs from cv. Sellie (A) and PC254K1 (B) inoculated with 7000* Ditylenchus africanus *at 150 days after planting. (Scale bars: A = 0.5 mm; B = 1 mm. DUM = damaged undifferentiated tracheary elements of the metaxylem, DP = differentiated tracheary elements of the protoxylem, EN = endocarp, M = mesocarp, UM = undifferentiated tracheary elements of the metaxylem.)*

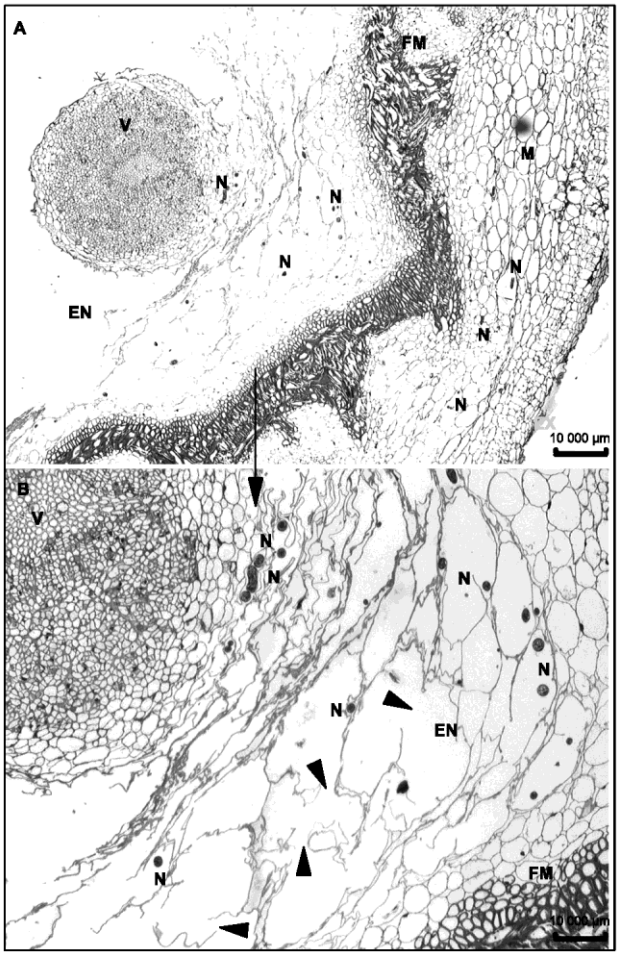

Fig. 4. *Light micrograph of a cross-section of a mature cv. Sellie pod inoculated with 7000* Ditylenchus africanus *specimens at 150 days after planting. A: The vascular bundle of the funicle and surrounding endocarp and mesocarp; B: The vascular bundle of the funicle and surrounding endocarp (arrows indicate collapsing of cells). Abbreviations: EN = endocarp, EX = exocarp, FM = fibrous mesocarp, M = mesocarp, N = nematode, V = vascular bundle of the funicle. (Scale bars: A= 10mm; B = 10mm.)*

ken 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).

Pods

From 90 **DAP** ***D.*** *africanus* was observed in pod tissue of cv. Sellie at all three inoculum levels. At 150 **DAP** several nematodes were observed in cv. Sellie pods at all three inoculum levels. However, no nematodes were observed in pods of cv. Sellie from the nematode-free control at 90 and 150 **DAP.** Cross-sections of mature pods of cv. Sellie 150 **DAP** inoculated with *ca* 7000 nematodes are presented in Figure 4. The funicle was surrounded by parenchyma cells of the endocarp (Fig. 4A). Adjacent to the endocarp the cells of the fibrous layer of the mesocarp were thick-walled. Parenchyma cells of the mesocarp are located between the fibrous mesocarp and the lignified layer of the exocarp. Nematodes were not present in the thick-walled fibres and tracheary elements of the vascular bundle of the funicle, in the thick walled cells

of the fibrous mesocarp or in the cells of the exocarp. However, they were observed in the parenchyma cells of the mesocarp as well as in those of the endocarp (Fig. 4A). The largest proportion of the nematodes present in cv. Sellie pods was observed around the vascular bundle of the funicle and in the parenchyma cells of the endocarp (Fig. 4B). Walls of the malformed, elongated parenchyma cells around the nematodes that were located in the endocarp around the vascular bundle were broken down and cells had started to aggregate. In advanced stages of parenchyma damage the cells completely collapsed (Fig. 4B). These parenchyma cells were not malformed, aggregated or collapsed in pod tissue of non-inoculated cv. Sellie plants from the nematode-free control.

Nematodes were observed in pods of PC254K1 for the 2000, 5000 and 7000 inoculum levels only at 90 DAP. No nematodes occurred in pods of PC254K1 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* 7000 nematodes are presented in Figure 5. The vascular bundles were located in the mesocarp adjacent to the fibrous mesocarp, which consists of thick-walled fibres. By contrast with the several numbers of nematodes observed in pod tissue of cv. Sellie, few nematodes were visible in the pod tissue of PC254K1 (Fig. 5). *Ditylenchus 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. 5B).

Differentiated tracheary elements of the protoxylem were thick-walled with a ribbed appearance similar to those in the peg tissue (Fig. 3). *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 opposed to those of inoculated cv. Sellie.

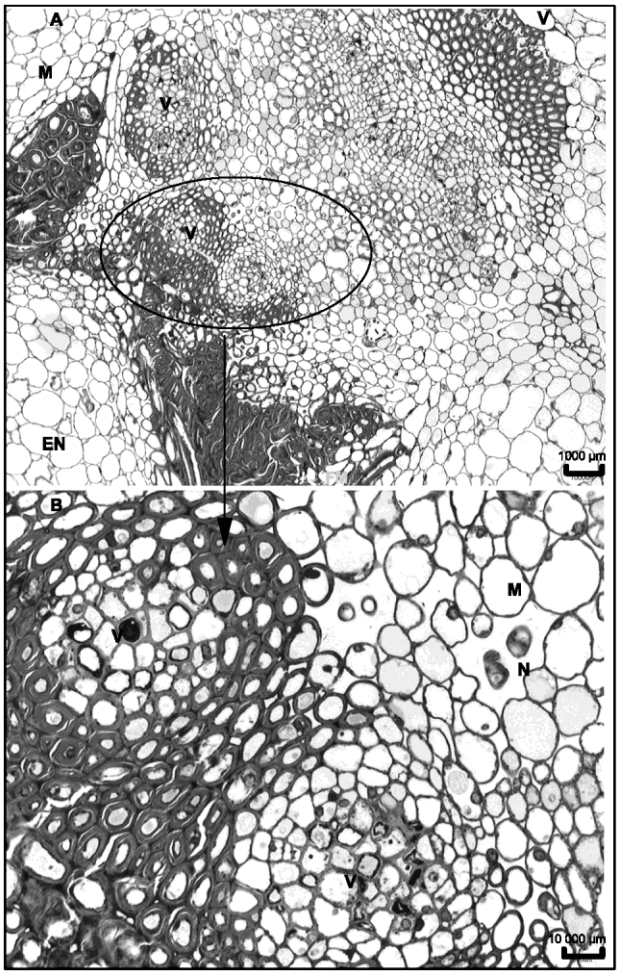

Fig. 5. *Light micrograph of a cross-section through a mature PC254K1 pod inoculated with 7000* Ditylenchus africanus *specimens at 150 days after planting. A: The vascular region and surrounding mesocarp; B: The vascular region. Abbreviations: EN = endocarp, FM = fibrous mesocarp, M = mesocarp, N =nematode, V =vascular bundles. (Scale bars: A= 1 mm; B = 10mm.)*

Discussion

Brown discolorations on pod surfaces and necrotic tissue at the connection point of pegs to pods observed on *D. africanus-infected* pods of cv. Sellie during this study were similar to those reported on the same cultivar in previous studies (Jones & DeWaele, 1988; DeWaele *et al.*, 1989). Contrary to the visible appearance of *D. africanus*-infected pods of cv. Sellie, those of inoculated PC254K1 showed no external damage symptoms associated with *D. africanus* infection. This supports results from previous screening studies (Steenkamp, 2009) that this groundnut genotype is resistant to *D. africanus* even at high nematode inoculum levels.

Absence of *D. africanus* from peg tissue of inoculated cv. Sellie of this study is in agreement with previous studies (Venter *et al.*, 1995). At this stage it is unclear what the cause of damage to undifferentiated tracheary elements of the metaxylem in the pegs of inoculated cv.

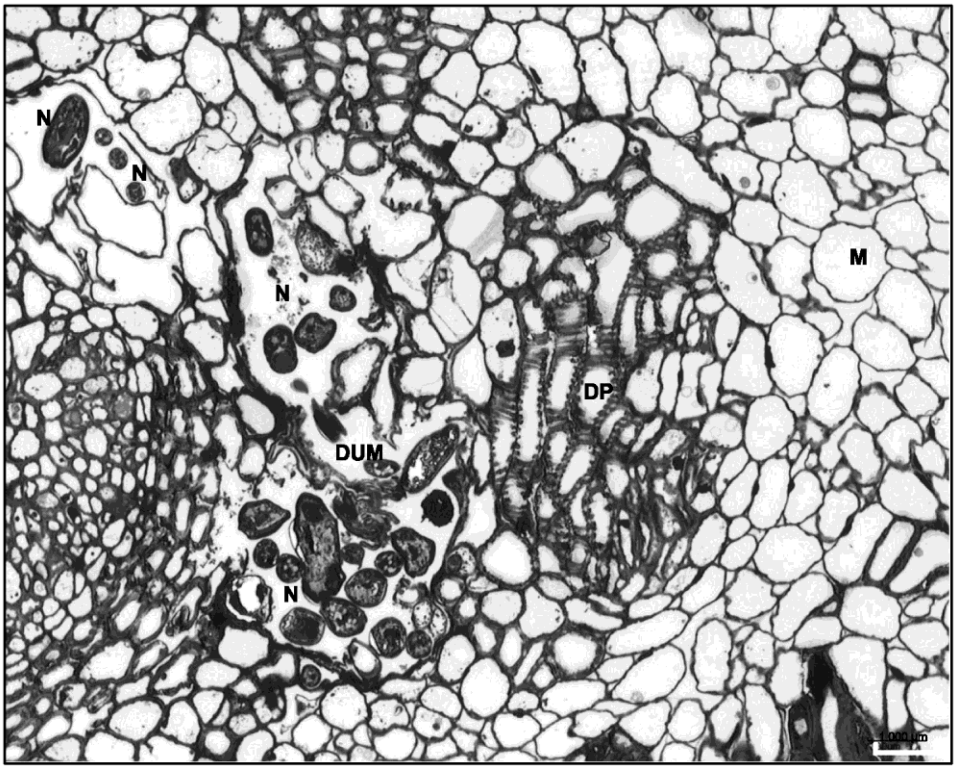

Fig. 6. *Light micrograph of a cross-section through the cells of the vascular region of a mature PC254K1 pod inoculated with 7000* Ditylenchus africanus *at 150 days after planting. Abbreviations: DUM = damaged undifferentiated tracheary elements of the metaxylem, DP =differentiated tracheary elements of the protoxylem, M = mesocarp, N =nematode. (Scale bar= 1 mm.)*

Sellie has been. The same applies to similar damage being visible in pegs of PC254K1 in the same region in peg tissue of their uninoculated counterparts.

The damage caused by ***D.*** *africanus* in infected, mature cv. Sellie pods at cellular level, as well as the presence of individual nematodes between the parenchyma cells of the mesocarp and endocarp but not beyond the mesocarp, support observations in previous studies (Jones & De Waele, 1988; DeWaele *et al.,* 1989). Jones and DeWaele (1990) and Venter *et al.* (1995) suggested that broken down cell walls as observed in our study are 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 cv. Sellie pods indicated that this might also apply to infection and migration caused by ***D.*** *africanus.*

Lack of damage and the absence of ***D.*** *africanus* nematodes in pod tissue of PC254K1 further indicate that the genotype is resistant to this nematode and suggest that the mechanism of resistance may be inhibition of proper development, migration and subsequent reproduction of ***D.*** *africanus.* The few nematodes that were visible in the region of the undifferentiated tracheary elements of the metaxylem during this study did not seem to induce cell collapse of the neighbouring parenchyma cells as proposed by Seinhorst (1957) and Castillo *et al.* (2007) for ***D.*** *dipsaci.* 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 (Timper *et al.,* 2007) is most probably the mechanism of

resistance involved. Additional research would be needed to establish whether production of pectolytic enzymes by D. *africanus* is involved as is the case with D. *dipsaci*. The cell wall chemistry of pods from PC254Kl attacked by *D. africanus* will also need to be studied in comparison with those of a susceptible host in order to determine the exact mechanism of resistance.

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