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Histological Studies of *Ditylenchus africanus* Within Peanut Pods[1]

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Abstract: Ditylenchus africanus entered the immature pegs and pods of peanut (*Arachis hypogaea* cv. Sellie) at the peg-connection and subsequently invaded the parenchymatous regions of the hull exocarp and endocarp, and eventually the seed testa. The nematode caused malformations of the cells of infected tissues, cell wall breakage, and cell collapse. The damage appeared to be due to enzymatic activity. In some testae the entire parenchyma region, which aids in protection of the seed, was destroyed. In immature pods, the nematodes moved across the fibrous region of the mesocarp into the hull endocarp. In mature pods, however, the fibrous mesocarp of the hull was lignified and apparently was a barrier to penetration of the inner pod tissues. In late-harvested pods, increased numbers of eggs and anhydrobiotes were found in the hull tissues, and eggs in the seed testa, suggesting the onset of winter survival mechanisms of the nematode.

Key words: Arachis hypogaea, Ditylenchus africanus, histology, nematode, peanut, penetration, survival mechanism.

Ditylenchus africanus sp. n. (15) causes blemishing and premature germination of peanut (*Arachis hypogaea* L.) seed before harvest, resulting in a lower grade of peanut yield and substantial economic losses to producers (13). The nematode, known previously as *D. destructor* (4), is found in all peanut production areas of South Africa. Seed infected with *D. africanus,* which is stored and then planted in uninfested soil the subsequent season, results in a downgraded yield (3).

While *D. africanus* is present in the roots of peanut and in the soil, 90% of the total population at harvest is found within the pods (1,14). Jones and De Waele (5) found that the nematode penetrates the peg near the point of connection to the pod, and from there it invades selected tissues of the hull and seed. These authors considered the associated tissue breakdown to be largely enzymatic, but did not describe the damage at the cellular level.

Ditylenchus africanus is able to enter a state of anhydrobiosis (3). One-third of such nematodes become active after rehydration to invade hulls and seeds of a new crop. Jones and De Waele (5) observed *D. africanus* in a coiled position on the surface of a cotyledon of a mature seed.

The objectives of the present investigation were to: 1) study the pod penetration by *D. africanus* over time, 2) describe the damage caused to the tissues of the hull and seed testa at the cellular level, and 3) determine if anhydrobiotes and eggs were present within these tissues.

Materials and Methods

Two trials were conducted in the greenhouse using nematode-free seeds of peanut cv. Sellie planted in 3-liter plastic pots (three seeds per pot) containing steam-sterilized sandy soil (93% sand, 4% silt, 3% clay). The seeds were coated with *Bradyrhizobium* sp. Seedlings were thinned to one per pot 14 days after planting (DAP). Plants were fertilized weekly with a nutrient solution (6.5% N, 2.7% P, 13% K), watered three times a week, and maintained at 20–25 C with a 13-hour photoperiod.

Inoculum of *D. africanus,* consisting of various life stages, was obtained from

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monoxenic cultures by macerating peanut leaf callus tissue (12). The plants were inoculated with *D. africanus* 21 DAP by pipetting the suspension into single, 50-mm deep holes impressed in the soil 10 mm from the base of the plant.

Hull and seed material of pods from uninfected plants and from infected plants showing symptoms of infection by *D. africanus* were collected, sectioned, and prepared for histological observations.

Experiment 1. Young pods: Twenty pots were inoculated with 1,000 *D. africanus* each, and 20 more retained as uninoculated controls. Pegs and immature pods were harvested at 49, 70, 91, and 112 DAP. Since the pods of a single plant varied in size and maturity, they were categorized according to age as follows: stage 1 = pegs without terminal swelling, stage 2 = pegs with pod swelling 2–3 mm in length, stage 3 = immature fleshy pods ca. 10 mm long, stage 4 = mature pods but with nonrigid hulls, and stage 5 = fully mature and rigid pods.

Pieces of peg, and tissue connecting the peg with the hull, from three pods of each age category, harvest date, and inoculum-level were fixed in formalin-alcohol-acetic-acid (FAA) for 24 hours at 24 C. The material was rinsed (three times for 15 minutes each) in both cacodylate buffer pH 7.4 (0.05 M) (10) and distilled water; dehydrated in an acetone series (50, 70, 90, 100, and fresh 100%, for 10 minutes each); processed through an acetone:Spurr's resin series (3:1, 2:1, 1:1, for 4 hours each) and embedded in fresh 100% Spurr's resin (8) for 8 hours at 70 C. Sections, 1-μm thick, were cut with glass knives on an ultra-microtome, floated on water on slides, dried on a hotplate, and stained with 0.5% toluidine blue in 1% borax (11). The sections were mounted with Entellan (Merck, Darmstadt, Germany) and examined using light microscopy.

Experiment 2. Mature hulls and seeds: Twenty pots were inoculated with 3,500 *D. africanus* each, and 20 more retained as uninoculated controls. Mature pods were harvested every 14 days from 133 to 189 DAP.

Pieces of hull and seed testa of three pods from each harvest date and inoculum-level were collected. Preliminary observations indicated that many nematodes in these mature tissues were in anhydrobiosis. A standard was established for comparison of active nematodes with those known to be in anhydrobiosis. This involved collecting pieces of fresh agar from cultures (12) containing active nematodes, and similar agar pieces from cultures dried slowly over a period of 3 months, containing anhydrobiotic nematodes. Plant and agar material was fixed in FAA for 24 hours at 24 C. The material was then dehydrated in a methyl cellosolve series and embedded in (2-hydroxyethyl)-methacrylat (GMA) in gelatin capsule shells according to the method of O'Brien and McCulley (7). Sections, 2-μm thick, were cut with glass knives on a rotary microtome, floated on water on slides, and air-dried. The material was stained by a modified Masson trichrome method (6) or, alternatively, with 1% toluidine blue (6). The sections were mounted with Entellan and examined using light microscopy.

Results

Experiment 1. Young pods: At 49 DAP, the oldest pods were at stage 2 development. The peg had a distinct exocarp, long vascular bundles, and a central pith surrounding a hollow core (Fig. 1A). Beneath the central pith of the peg a layer of cells in the pericarp (hull) had begun to differentiate to form the fibrous mesocarp layer. These cells were small and thin-walled. All tissues stained blue, indicating a predominance of cellulose in the material. No nematodes were observed in stage 2 pods or in the pegs (stage 1) of plants from inoculated pots.

At 70 DAP, the oldest pods were at stage 3. Cell-wall thickening of some of the cells forming the fibrous mesocarp layer of the hull was evident (Fig. 1B). Regions of thin-

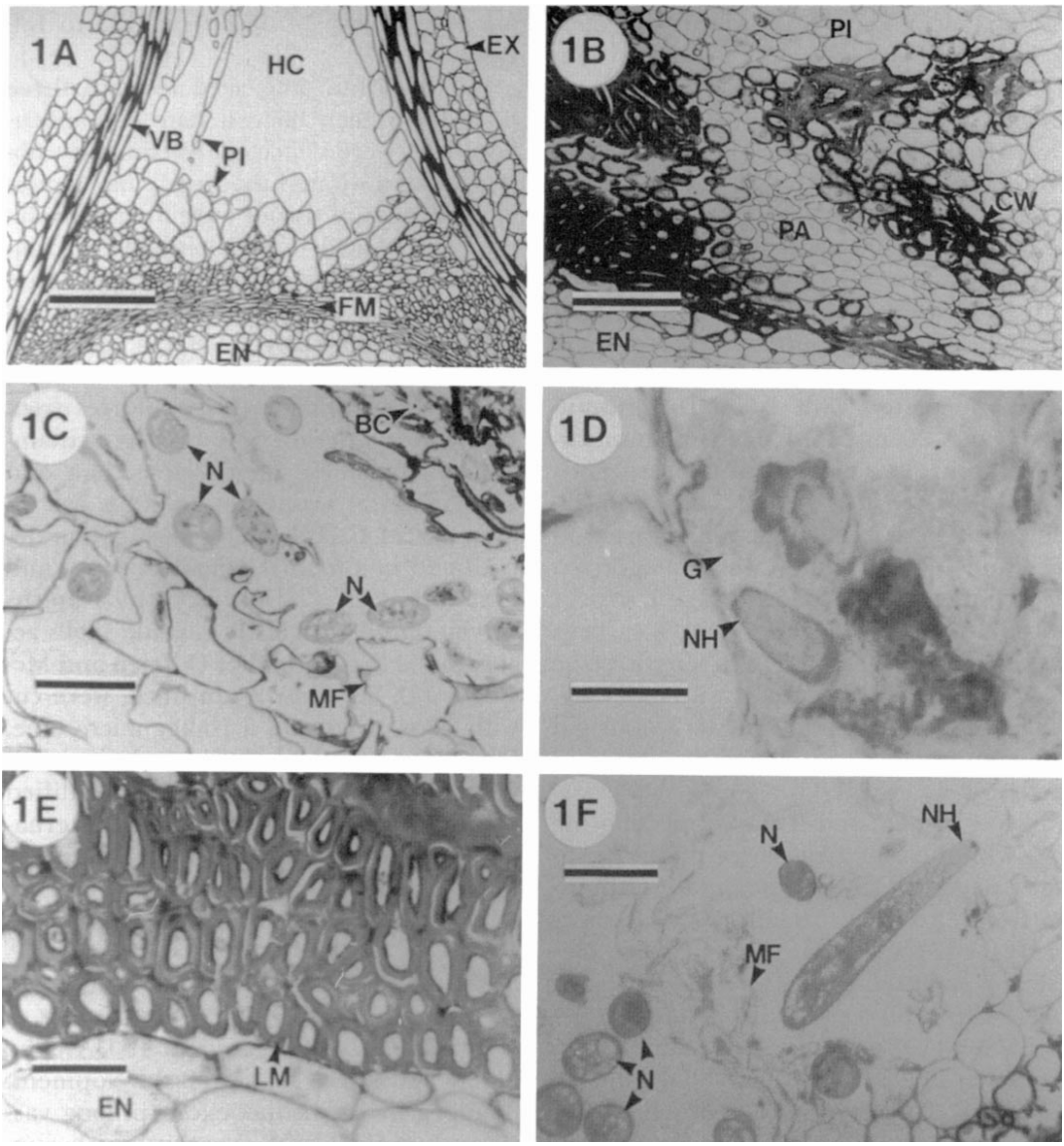


FIG. 1. Longitudinal section A) Drawing of peg-connection of stage 2 peanut pod at 49 days after planting (DAP); B) Immature fibrous mesocarp of stage 3 peanut hull at 70 DAP; C) *D. africanus* in exocarp at shoulder between peg and hull (stage 4) at 91 DAP; D) *D. africanus* in exocarp of stage 2 peg at 91 DAP; E) Mature fibrous mesocarp of stage 5 peanut hull at 112 DAP; F) *D. africanus* in endocarp of stage 5 peanut hull at 112 DAP. Scale bar A) 400 μm; B) 100 μm; C) and E) 50 μm; D) 20 μm; F) 30 μm. Sections stained with toluidine blue. BC = broken cell walls; CW = thickened cellulose cell walls; EN = endocarp of hull; EX = exocarp; FM = fibrous mesocarp of hull; G = granules; HC = hollow core of peg; LM = lignified mesocarp cells; MF = malformed cells; N = nematode; NH = nematode head; PA = path of thin-walled cells; PI = pith of peg; VB = vascular bundle.

walled cells remained between the thicker-walled cells, and all tissues stained blue. A nematode section was visible between broken cell walls (similar to symptoms observed in Fig. 1C) of the pith at the periphery of the hollow core of the peg from an infected plant. Similar cell walls from uninfected plants were not broken. Again, nematodes were not visible in stage 2 pods or stage 1 pegs.

At 91 DAP, the pods were at stage 4 development. A greater number of cells in the mesocarp of the hull were thicker-walled, but traces of thin-walled cells (observed in Fig. 1B) were still present. Nematode sections were found in the exocarp at the shoulder between the peg and hull (Fig. 1C). The cells in this area were malformed and the cell walls broken. These characteristics were not present in the same tissue in pods from uninfected plants. In the stage 3 pods, nematode sections were seen in the hollow central core of the peg or between broken cell walls of the pith on the periphery of the core. The cell walls in this region in pods from uninfected plants were not broken. In the stage 2 pods, nematodes were found in the periphery of the hollow core and in the exocarp of the peg. The cells in the latter region were broken, and granules were aggregated around a nematode head (Fig. 1D). Farther away from this site, similar granules aggregated against the intact cell walls and nuclei. Broken cell walls and extracellular granular aggregations were not found in this tissue of the pods from uninfected plants.

At 112 DAP, the pods were at stage 5, and the cells of the mesocarp of the hull were all thick-walled (Fig. 1E). These cells stained green-blue, indicating impregnation of the cellulose material with lignin. Nematode sections were numerous in the exocarp and hollow core of the peg, and in the endocarp of the hull. The endocarp cells surrounding a group of nematodes, including a nematode head, were malformed with long and convoluted cell walls and had begun to collapse (Fig. 1F). These cells in pods from uninfected plants were not malformed.

Experiment 2. Fresh and dried agar: The microscopic structure of the body of the anhydrobiotic *D. africanus* (Fig. 2A) from dried agar differed markedly in two respects from those of the active nematodes (Fig. 2B) from fresh agar. The body contents of the anhydrobiotic nematodes appeared dense, stained darkly, with the hypodermis thin and often not distinguishable from the body contents. In contrast, the body contents of the active nematodes stained lightly, while a thick body wall and cuticle stained darker. In the anhydrobiotic nematodes, the globular structures observed in most sections were small (0.5 to 1.0 μm) and densely arranged, whereas in the active nematodes they were larger (diameter variable, 2 to 4 μm). These structures appeared to be situated within the intestine and were not seen in the head, gonadal regions, or tail.

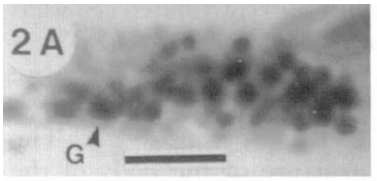
Experiment 2. Mature hulls: In the peanut hull (Fig. 3A), *D. africanus* was found in the endocarp, parenchymatous region of the mesocarp, and occasionally in the cork layer of the exocarp. These tissues stained blue with toluidine blue, indicating the presence of cellulose. Nematodes were not seen in the fibrous region of the mesocarp, which stained green with toluidine blue and indicated a high lignin content. Nematodes were also not found in the thick-walled cells of the vascular bundles.

No *D. africanus* was observed in the exo-

FIG. 2. Longitudinal section of A) Anhydrobiotic *D. africanus* in agar dried over 3 months; B) Active *D. africanus* in fresh agar. Scale bar A) and B) 5 μm (in A). Sections stained with toluidine blue. BW = body wall; G = lipid globules.

FIG. 3. Cross section A) Drawing of peanut hull; B) and C) *D. africanus* in fragmented parenchymatous mesocarp of the hull. Scale bar A) 200 μm; B) 75 μm; C) 50 μm. Sections stained with Masson trichrome. EN = endocarp; EX = exocarp; LM = lignified mesocarp; N = nematode; PM = parenchymatous mesocarp; VB = vascular bundle.

FIG. 4. Cross section A) Drawing of peanut seed testa; and B) Parenchyma of testa that is severely fragmented. C) Longitudinal section of *D. africanus* in tunnel formed by collapsed parenchyma, immediately beneath the epidermis of the testa. Cross-sections of D) First-stage juvenile *D. africanus* curled within egg shell; E) Adult *D. africanus* with distinct body wall. Scale bar A) and B) 50 μm (in B); C) and D) 15 μm; E) 30 μm. Sections stained with Masson trichrome. BW = nematode body wall; CP = collapsed parenchyma; EN = endosperm; EP = epidermis; ES = egg shell; N = nematode; P = parenchyma tissue; T = tunnel; VB = vascular bundle.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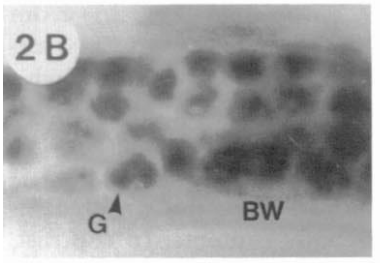
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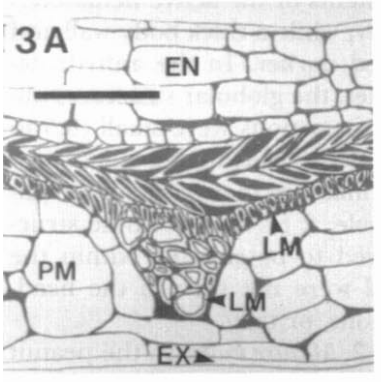
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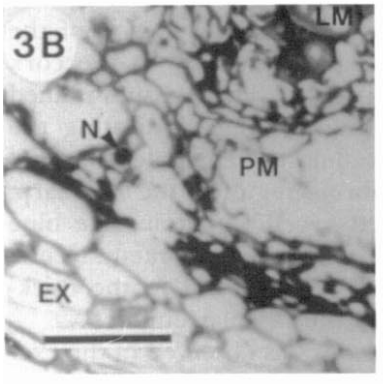
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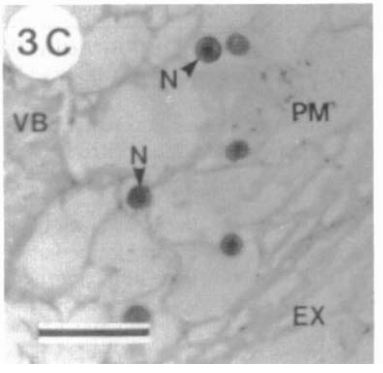
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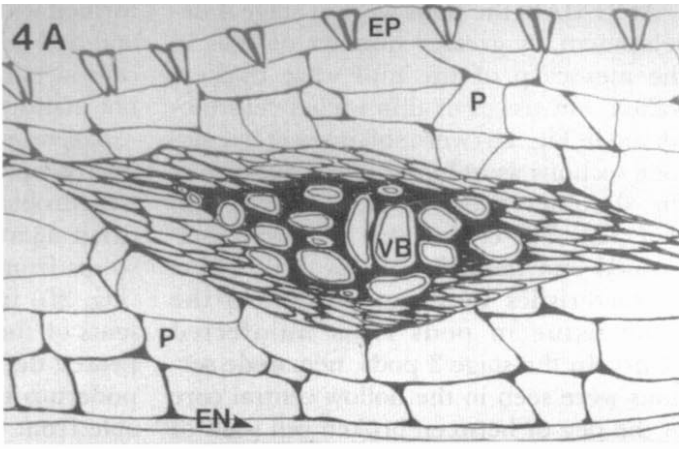
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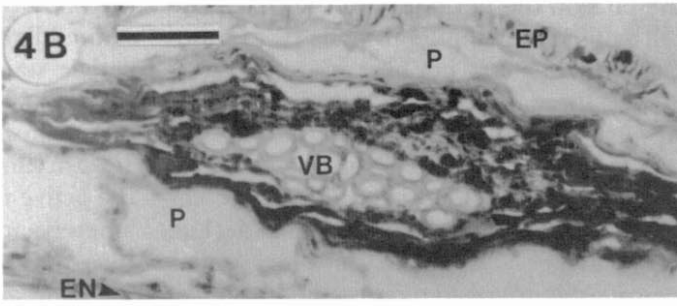
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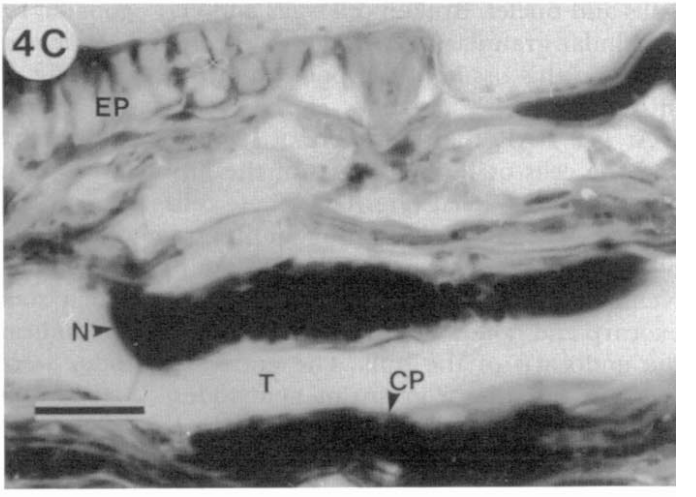
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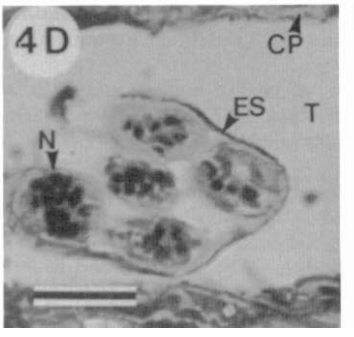
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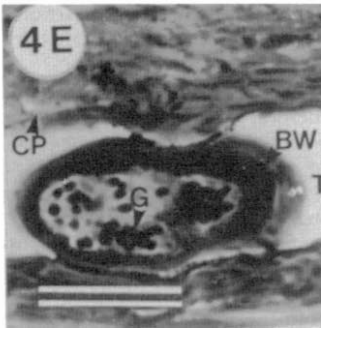
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carp of early harvest (133 DAP) samples. Many nematodes were, however, present in the parenchymatous mesocarp, which was often fragmented (Fig. 3B). This tissue was never fragmented in hulls of uninfected plants. The body structure of the nematodes exhibited a distinct body wall and large globular structures. Eggs, some of which contained first-stage juveniles, were also observed in the parenchymatous mesocarp. Many nematodes with distinct body walls were found in the fragmented endocarp. In the most mature pods, the endocarp was partially compressed against the fibrous mesocarp, and fewer nematodes were found in this tissue.

At normal harvest (147 DAP), nematodes with distinct body walls were found in the exocarp. Most of the nematodes in the parenchymatous mesocarp (Fig. 3C) and endocarp exhibited a distinct body wall and large globular structures, but some had no distinct body wall and had small globular structures. At 161 DAP, nematodes without a distinct body wall were seen in the exocarp. Eggs were also observed in the parenchymatous mesocarp and fragmented endocarp.

In late-harvest samples (175 and 189 DAP), the cells of the exocarp were collapsed and only a few nematodes with no distinct body wall were visible. At this stage the parenchymatous mesocarp had naturally pulled away from the fibrous mesocarp, and the nematodes were usually found in the resultant spaces. These nematodes and the small number found in the endocarp were without a distinct body wall.

Experiment 2. Mature seed testa: In the peanut seed testa (Fig. 4A), *D. africanus* was always found in the parenchyma region. As in the hulls, the nematode was never found in the vascular tissue.

In the early-harvest (133 DAP) and normal-harvest-time samples (147 DAP), large numbers of *D. africanus* were observed in the parenchyma layer. The nematodes exhibited a distinct body wall and large globular structures. The parenchyma cells showed little or no damage.

At 161 DAP, fewer nematodes with distinct body walls were observed. The parenchyma was severely fragmented, collapsed, or was often conspicuously absent (Fig. 4B). The vascular bundles were intact and often surrounded by collapsed parenchyma. The parenchyma was intact in the testae from uninfected plants.

In the late-harvest samples (175 DAP), the nematode body walls were distinct (Fig. 4C). Eggs (Fig. 4D) were also first observed in mature testae at this time. The parenchyma was often present, but was fragmented and collapsed. At 189 DAP, large numbers of nematodes and eggs were present. Most nematodes had distinct body walls (Fig. 4E), and the parenchyma was fragmented or absent.

Discussion

Ditylenchus africanus was most often found in the parenchymatous tissues of the exocarp of the immature peg and pod, in the central pith and hollow core of the peg, and in the endocarp of the hull. In the mature pods, nematodes were found in the parenchymatous tissues of the hull endo-, meso-, and exocarp, and in the parenchyma of the mature seed testa. The damage to the seed testa was particularly severe. The nematode was not found in the thick-walled vascular tissue of either the hull or seed testa. This is in contrast to the observations of Jones and De Waele (5), who found the nematode in the vascular tissue of the seed testa of the same cultivar. It appears, however, that the diameter of the vascular cells in both the hull and seed testa are too narrow to permit infection.

The nematode was also not found in the lignified hull mesocarp, and there was no evidence that the nematodes moved from the parenchymatous mesocarp, through the lignified layer, into the endocarp. However, it was apparent that in younger pods the nematodes were able to penetrate the immature mesocarp, possibly through the regions of parenchymatous cells, and enter the endocarp of the hull. Infection

of the hull endocarp of lignified pods was probably established before 91 DAP. From this site, the nematodes infect the peanut seeds through the micropyles (5). If infection occurs after the mesocarp of the hull is fully lignified, it is unlikely that *D. africanus* is able to penetrate the endocarp and seeds. There is supporting evidence for this. Basson et al. (2), reported that plants infected with 500 nematodes at 105 DAP yielded high-quality seeds. Plants infected before 150 DAP yielded seed of poor quality.

In contrast, the tissues of the peg are continuous with the outer tissues of the hull. Nematodes found in the peg can probably migrate through and damage these hull tissues. The gray and bruise-like (13) pod disease symptoms may result from damage to the outer parenchymatous mesocarp.

Symptoms of damage by *D. africanus* were observed at the cellular level. Cell walls appeared to be damaged by enzyme secretions. The cells were smaller than neighboring healthy cells and probably in an advanced stage of senescence. At other sites, cell walls were broken and a granular aggregation was visible around the nematode head. The identity of these granules is uncertain, but may be the disarranged protoplasm from destroyed cells. In advanced infections, cells collapsed completely.

Peanut yield is negatively affected by premature seed germination before harvest, and by an increased percentage of unsound kernels (13). Premature germination is prompted by destruction of the testa and the resultant rapid leaching of germination inhibitors (9). Hull breakdown allows increased water seepage and also facilitates germination. The breakdown of the testa may result in splitting of seed, which decreases peanut grade and results in poor plant population densities.

Differences between anhydrobiotic and active nematodes have been described in studies of the rehydration of *Ditylenchus dipsaci* juveniles. Thickening of the hyaline layer beneath the cuticle in active nematodes, suggesting a repair in the muscle cells in readiness for reactivity, and the coalescence of lipid droplets within the intestinal cells were observed in activated nematodes (16,17). The latter probably explains the larger globules found in the active nematodes of this study.

Many eggs and anhydrobiotes were observed in the hull tissues of cv. Sellie from 161 DAP, and in low numbers as early as 133 (eggs) and 147 (anhydrobiotes) DAP. It appears that eggs and anhydrobiotes are involved in the winter survival of *D. africanus* in decaying hulls and stubble. Removal of the decaying pods from the field at harvesting, when possible, should reduce the level of infestation of this pest. Shelling in the field may lead to repeated field infestations.

Since many eggs were found in the testa of cv. Sellie at late harvest, and few anhydrobiotic nematodes were observed, it may be concluded that eggs are an important winter survival stage in stored seed. Infected seed cold-stored for 5 months before planting can result in an infected harvest the following season (3). Tactics to reduce the number of eggs in seed will help control the pest on future peanut crops. This may include timely harvesting and should be a requisite in the production of certified plant seed.

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