Development of *Heterodera avenae* Woll. and host cellular responses in susceptible and resistant wheat

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Summary – Juvenile development of the cereal cyst nematode (*Heterodera avenae* Woll.), and the anatomy and ultrastructure of nematode-induced syncytia in wheat *(Triticum aestivum L.)* were studied using the near isogenic lines Prins (susceptible) and AUS 10894 × Prins (resistant). After synchronised inoculation, male development proceeded to completion in both cultivars, while female development was arrested at the fourth stage in the resistant cultivar. Syncytia were initiated in both cultivars by day four, and their development appeared similar until day fifteen. At this stage syncytia in the susceptible cultivar became highly active in appearance, vacuoles decreased in size, and an increased volume of cytoplasm had numerous organelles. Syncytia in the resistant cultivar at day fifteen were dominated by coalescing large vacuoles, with reduced cytoplasm. By day 33 syncytial cytoplasm was completely degenerate. Endodermal necrosis was observed in both cultivars. The timing of cellular responses is discussed.

Résumé – *Développement d'*Heterodera avenae *Woll. et réactions au niveau cellulaire des blés sensibles et résistants.* Le développement des juvéniles du nématode à kystes des céréales (*Heterodera avenae* Woll), ainsi que l'anatomie et l'ultrastructure des syncitiums induits chez le blé par le nématode, sont étudiés sur les lignées isogéniquement proches Prins (sensible) et AUS 10894 × Prins (résistante). Après inoculations synchrones, le développement des mâles va jusqu'à son achèvement chez les deux cultivars tandis que celui des femelles est stoppé au quatrième stade juvénile chez le cv. résistant. Chez les deux cultivars, la formation des syncitiums débute au quatrième jour et ils se développent de façon identique jusqu'au quinzième jour. A ce stade, chez le cultivar sensible les syncitiums deviennent visiblement très actifs, la taille des vacuoles diminuant tandis que le volume du cytoplasme augmente et que de nombreux organites apparaissent. Chez le cultivar résistant, les syncitiums sont caractérisés par la coalescence de volumineuses vacuoles et la réduction du cytoplasme. Au trente-troisième jour, le cytoplasme du syncitium est complètement dégénéré. Une nécrose de l'endoderme est observée chez les deux cultivars. La chronologie des réactions cellulaires est discutée.

Key-words : Nematodes, wheat, *Heterodera avenae*, ultrastructure, syncytia.

Heterodera avenae Wollenweber, the cereal cyst nematode (CCN), is widely distributed throughout the world's cereal growing areas (Meagher, 1977), and is the major wheat root pathogen in southern Australia (Brown, 1984). CCN biology (Fisher, 1981), and its effect on cereal growth (Volkmar, 1989) have been studied, although little is known about the development of the nematode within host roots. Resistance to CCN in cultivars of wheat, barley and oats has been observed (O'Brien & Fisher, 1974) but the mechanisms of resistance have not been identified. The juveniles of cyst nematode genera invade the tips of host roots and migrate intracellularly through the cortex until they settle permanently at their feeding site, a syncytium induced by the nematode (Dropkin, 1969), which resembles the transfer cells of plants described by Pate and Gunning (1972). The cells of syncytia are modified to sequester nutrients from the host plant vascular system for ingestion by the nematode (Jones, 1981). Cellular modifications include cell hypertrophy, main vacuole reduction, cytoplasmic organelle proliferation, and wall ingrowth formation (Jones & Northcote, 1972a, b).

The ultrastructure of interactions between various sedentary nematode species and their compatible and resistant hosts has been investigated (Endo, 1991). Several different resistant host responses have been observed : a hypersensitive reaction that appears to isolate the syncytium (Paulson & Webster, 1970), the deterioration of the syncytium following its successful establishment (Gipson *et al.,* 1971, Riggs *et al.,* 1973, Grymaszewska & Golinowski, 1987), or the resistance of cells to syncytial incorporation (Magnusson & Golinowski, 1991).

This study aims to investigate the cellular structure of nematode-induced syncytia in near-isogenic cultivars of wheat, one of which carries a CCN resistance gene from the cultivar AUS 10894, to gather information on how and when the resistance mechanism may be operating.

Materials and methods

Roots of wheat (*Triticum aestivum* L.) near-isogenic cultivars AUS 10894 × Prins (seven backcross generations, resistant) and Prins (susceptible) were examined. Surface-sterilised grains were germinated on 1 % agar in the dark at 20 °C for 48 h. The emerging roots

were then inoculated with approximately 50 juvenile cereal cyst nematodes *(H. avenae)* of the Australian pathotype (Andersen & Andersen, 1982). Second stage juveniles were obtained from cysts extracted from soil and hatched at 10 °C. The juveniles were surface-sterilised in 0.125 % Penicillin-G, 0.125 % Streptomycin sulphate and 0.0125 % Tetracycline hydrochloride overnight, and resuspended in sterile distilled water at a concentration of 2500 per ml. The inoculum (20 μl) was pipetted onto the surface of the agar immediately adjacent to the root tips. After two days the seedlings were removed from the inoculation plates to prevent further penetration and to ensure synchronised infection, and were transferred to 100 ml of B & D medium (Kondorosi *et al.*, 1984) in 500 ml containers at 15 °C with a 12 h day : night photoperiod.

Root samples were taken at various times from two to 36 days after inoculation for light and transmission electron microscopy. Sections of primary root (approx. 3 mm) containing a nematode-induced gall and associated lateral roots were pre-fixed overnight in 3 % gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7). Tissues were post-fixed for 3 h in 1 % osmium tetroxide and for 30 min in 0.5 % uranyl acetate, prior to dehydration in acetone and embedding in Spurrs resin. For light microscopy on a Zeiss axiophot microscope, thick (3 μm) transverse and longitudinal sections were cut on a microtome, stained with 0.1 % methylene blue and 0.1 % azure II in 1 % borax solution (Richardson *et al.*, 1960), and mounted in DPX. For transmission electron microscopy on a Philips TEM, 70 nm sections cut on a LKB ultramicrotome were picked up on Formvar-coated grids and stained for 12 min in 0.4 % uranyl acetate and for 8 min in Reynolds lead citrate (Reynolds, 1963). For nematode counting and size measurement, roots were stained with 0.05 % acid fuchsin in lactoglycerol (Bridge *et al.*, 1982). Body sizes were determined by measuring the plan area of drawings made with a camera lucida with an OTT planimeter. Approximately twenty individuals were measured at each time point.

Results

During all experiments, the susceptible and resistant cultivars were treated similarly, and nematode development on the susceptible cultivar demonstrated that the axenic culture system did not affect the ability of juveniles to penetrate roots and develop fully. Most nematodes had made their way onto the surface of the root tip by 24 h post inoculation, and had penetrated the root tip within 48 h, at which stage the seedlings were transferred to fresh media. Following inoculation, the region of the root immediately behind the root apical meristem became densely populated by root hairs resulting from the inhibition of root elongation by the nematodes. This reaction signalled syncytial initiation and these regions swelled into galls within four days and produced lateral roots within 14 days. When few juveniles penetrated the

roots gall formation was slower or failed to occur, so that the time of appearance and the size of galls indicated the efficiency of inoculation. The synchronised infection system and gall size selection were therefore used to examine root sections containing nematodes and root tissues of similar age and density.

Figure 1 shows the increase in nematode body size for juveniles in the resistant and susceptible cultivars. Juveniles in both cultivars increased in size at the same rate until their third moult at seventeen days. At this stage males had ceased feeding. Females on the susceptible cultivar increased in size rapidly after 20 days, while growth of those in the resistant cultivar was greatly reduced, so that they failed to develop to maturity, giving an unequal final sex ratio.
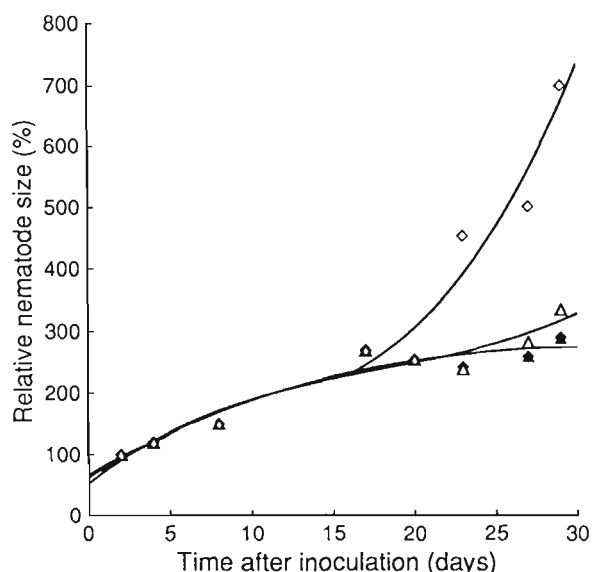

Fig. 1. The body size of *Heterodera avenae* in susceptible and resistant wheat cultivars at various times, expressed as a percentage of its body size at two days post-inoculation. (◊ : females and ♦ : males in the susceptible cultivar; △ : females and ▲ : males in the resistant cultivar).

Figure 2 presents cellular responses to *H. avenae* juvenile invasion of roots of the susceptible wheat cultivar Prins. By day four, the juveniles had migrated towards the centre of the root, settled against the endodermis, and initiated their permanent feeding site in the stele. The feeding site, a syncytium, is formed by cell wall dissolution of pericycle and vascular parenchyma cells, in some cases it may incorporate immature metaxylem (Fig. 2 A). Necrotic areas appeared at an early stage between the nematode body and the syncytium as endo-
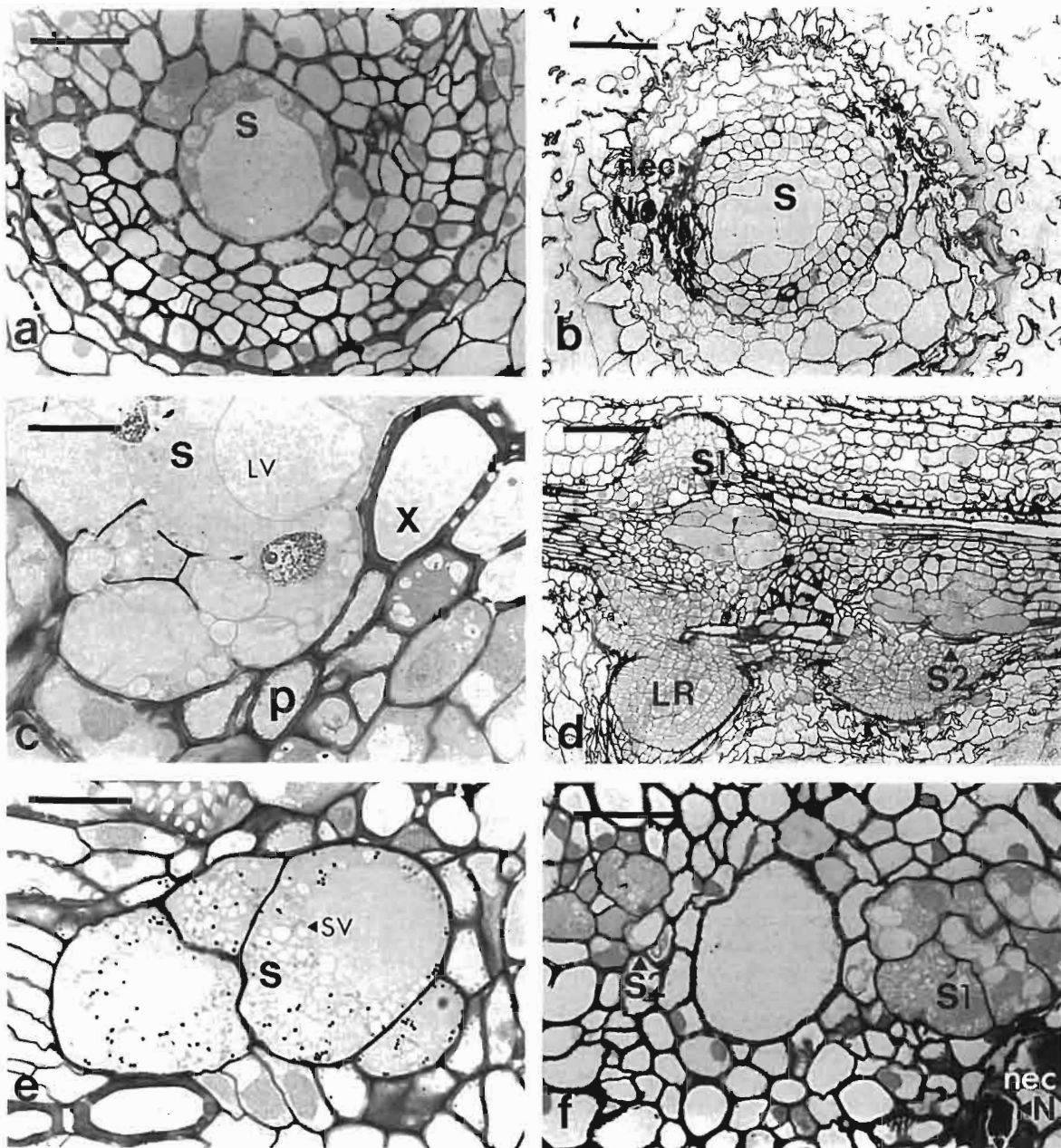
Fig. 2. Structure of syncytia induced in the roots of the susceptible wheat cultivar Prins by *Heterodera avenae*. A : Transverse section (TS) of day four syncytium incorporating central metaxylem element; B : TS of day eight stele containing large syncytium, with extensive arc of necrosis between the nematode and syncytium; C : TS of day eight syncytium showing large vacuoles and close contact with xylem and phloem; D : Longitudinal section (LS) of two fifteen-day-old syncytia, showing appearence of small vacuoles, multiple lateral meristems, and disorganised vascular tissue; E : TS of day nineteen syncytium, showing many small vacuoles, and larger vacuoles filled with amorphous material. Cytoplasm stains intensely, and contains osmophilic granules; F : TS of two day 21 syncytia. Few large vacuoles remain, nuclei are hypertrophied, and cytoplasm stains intensely. Necrosis is evident around nematode head. S, S 1, S 2 = syncytia, N = nematode, nec = necrosis, LV = large vacuole, SV = small vacuole, x = xylem, p = phloem. *(Bar equivalents : A, F = 50 μm; B = 100 μm; C = 20 μm; D = 200 μm; E = 25 μm.)*

dermal cells collapsed. This necrosis sometimes extended in an arc around the vascular cylinder, but was not observed between the syncytium and vascular cells (Fig. 2 B).

At day eight, the syncytium was still characterised by extensive vacuolation (Fig. 2 C). By day fifteen, a major change occurred in the appearance of the syncytium in the susceptible cultivar, as vacuoles became small and numerous, with a corresponding increase in cytoplasmic volume. The cytoplasm stained intensely, and the large vacuoles were clear or filled with an amorphous material (Fig. 2 D). Lateral roots were initiated, and vascular elements were forced into irregular arrangements to avoid these and the large syncytia (Fig. 2 D). Osmophilic granules appeared in the cytoplasm of very active syncytia (Fig. 2 E). By day seventeen, syncytia were highly active, with hypertrophied nuclei and few large vacuoles, even when the nematode head was surrounded by necrotic cells (Fig. 2 F).

Figure 3 shows root cell responses to juvenile invasion in the resistant wheat cultivar AUS 10894 × Prins. By day four, syncytia were established. They had large vacuoles containing a granular material, and a small amount of cytoplasm, differing in appearance from other vascular parenchyma cells (Fig. 3 A). As in the susceptible cultivar, the endodermis adjacent to the syncytium become necrotic (Fig. 3 A, B). Cells incorporated into the syncytium were hypertrophied, their walls partially digested, leaving wall fragments which indicated their original shape (Fig. 3 B). The syncytium was multinucleate, nuclei were hypertrophied with scattered chromatin. The syncytium extended longitudinally along the axis of the root, and had extensive contact with vascular elements (Fig. 3 B). At eight days post-inoculation, the syncytium was still similar in appearance to earlier stages (Fig. 3 C). Wall dissolution continued as the syncytium integrated more cells, but the outer wall of the syncytium was unaffected, and was thickened where it was adjacent to the nematode (Fig. 3 C, D). By fifteen days, syncytia in the resistant cultivar were still mainly dominated by large vacuoles, with weakly staining cytoplasm, indicating low activity (Fig. 3 D). Proliferation of coalescing vacuoles in the syncytium of the resistant cultivar by nineteen days greatly reduced the volume of cytoplasm (Fig. 3 E). At thirty-three days, syncytia may be totally nonfunctional, with complete tonoplast breakdown (Fig. 3 F).

Figure 4 shows the ultrastructure of syncytia in resistant and susceptible wheat cultivars. Syncytia in the susceptible cultivar had dense cytoplasm, with numerous mitochondria, plastids, endoplasmic reticulum, free ribosomes and small vacuoles (Fig. 4 A). Membrane-bound wall ingrowths were found adjacent to the nematode feeding site and vascular elements in mature syncytia (Fig. 4 B). In contrast, syncytia in the resistant cultivar had cytoplasm reduced to a thin layer around the cell walls and wall fragments (Fig. 4 C). Cell organelles were compressed into this thin layer of cytoplasm (Fig. 4 C). In the later stages of syncytial breakdown cellular membranes lost their integrity, with cytoplasm and chromatin dispersing (Fig. 4 D).

Discussion

This study has shown that *H. avenae* juveniles successfully initiate feeding sites in resistant and susceptible wheat cultivars. Potential females in the susceptible cultivar undergo a massive increase in size in the later stages of their development, while in the resistant cultivar they fail to develop beyond the fourth stage. The most significant cellular difference observed between the cultivars was seen at about fifteen days, when the syncytia of the susceptible cultivar became highly active with increased cytoplasmic volume. In contrast, the syncytium in the resistant cultivar was less active and highly vacuolated. Up till this time, syncytial development appeared to be the same in both cultivars.

Within four days of penetrating the primary roots, juveniles had initiated a feeding site or syncytium, which is believed to be induced by the nematode stylet injecting secretions into an initially modified pericycle cell (Rice *et al.*, 1987). The cytoplasm of this cell then became confluent with vascular parenchyma cells as they were incorporated by wall degradation. The syncytium extended longitudinally along the root axis and had good contact with vascular elements along its length in both cultivars, in contrast with *H. schachtii* induced syncytia in *Sinapsis alba*, where in the resistant cultivar contact with the xylem was prevented by the necrosis of xylem parenchyma (Magnusson & Golinowski, 1991). In wheat roots a zone of necrotic cells was often observed between the nematode and its syncytium, where cells of the endodermis had broken down and become electron dense. This response appears to be similar to the necrosis noted in other nematode interactions (Endo, 1991). But it occurs in both the resistant and susceptible cultivars and it does not completely isolate the syncytium, nor does it appear to hinder syncytial development and nematode feeding. The necrosis may therefore be part of a non-specific endodermal response to a pathogen being detected near the stele, and in this case is ineffective against the nematode.

Syncytia in the susceptible cultivar at later stages are characterised by reduced vacuole volume, active cytoplasm with numerous organelles, and wall ingrowths. The wall ingrowths and wall thickenings are found where the syncytium abuts vascular elements and the nematode head, and provide enlarged membrane surface area for enhanced transport into and out of the syncytium. They are formed as a result of nematode demand for nutrients (Jones & Northcote, 1972*b*), and are characteristic of transfer cells (Pate & Gunning, 1972).
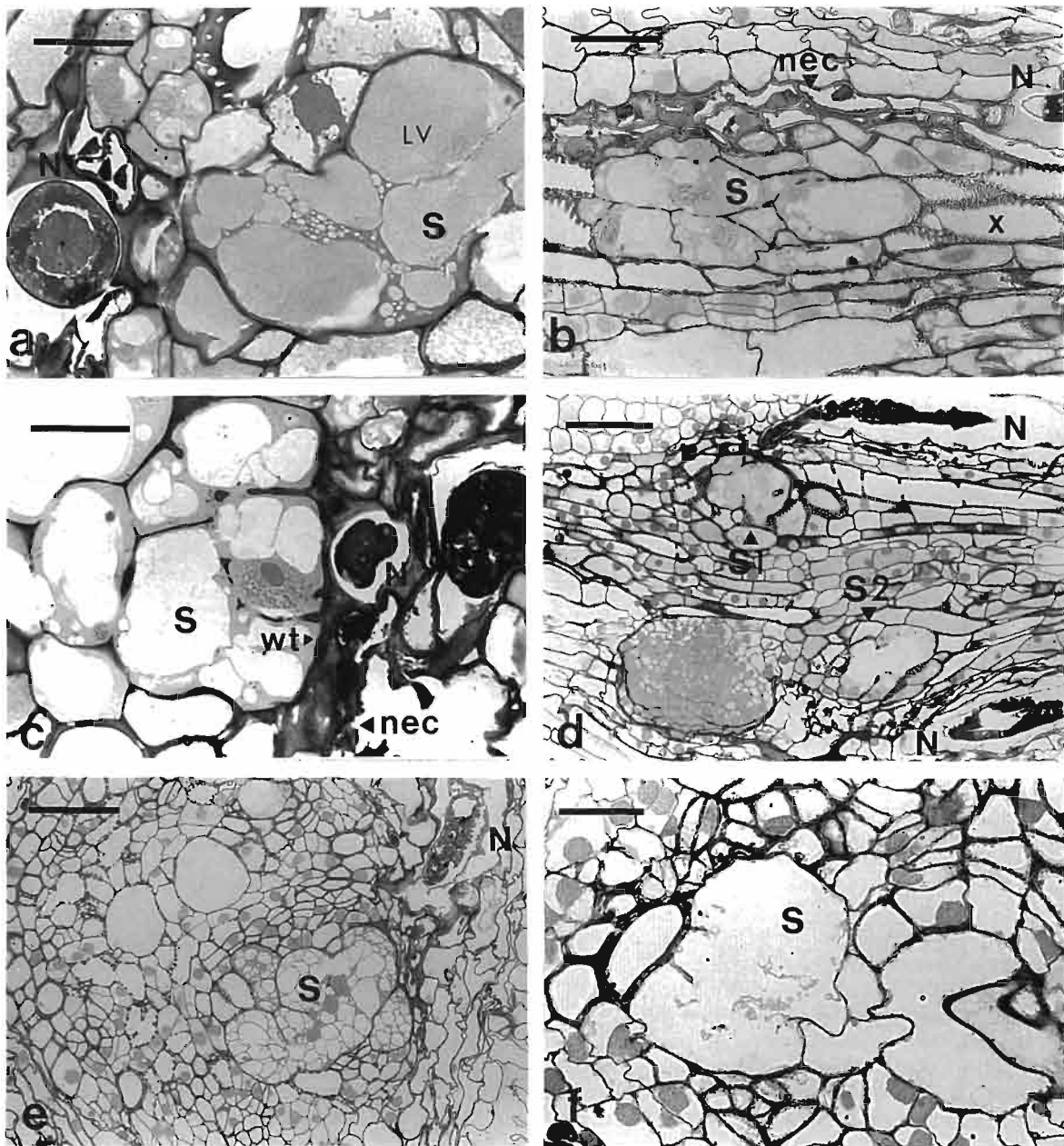
Fig. 3. Structure of syncytia induced in the roots of the resistant wheat cultivar AUS 10894 × Prins by *Heterodera avenae*. A : TS of day four syncytium, and vascular parenchyma cells; B : LS of day four syncytium, showing hypertrophied cells, cell wall breakdown, and large vacuoles. Endodermal cells adjacent to syncytium are necrotic; C : TS of day eight syncytium with large vacuoles and wall thickening adjacent to nematode; D : LS of two day fifteen syncytia and associated juveniles. Syncytial cytoplasm stains weakly; E : TS of day seventeen syncytium, showing predominance of large coalescing vacuoles; F : TS of day 33 syncytium, showing complete cytoplasmic degeneration. S, S 1, S 2 = syncytia, N = Nematode, nec = necrosis, LV = large vacuole, wt = wall thickening, x = xylem. *(Bar equivalents : A, C = 25 µm; B = 50 µm; D = 100 µm; E = 200 µm; F = 35 µm.)*
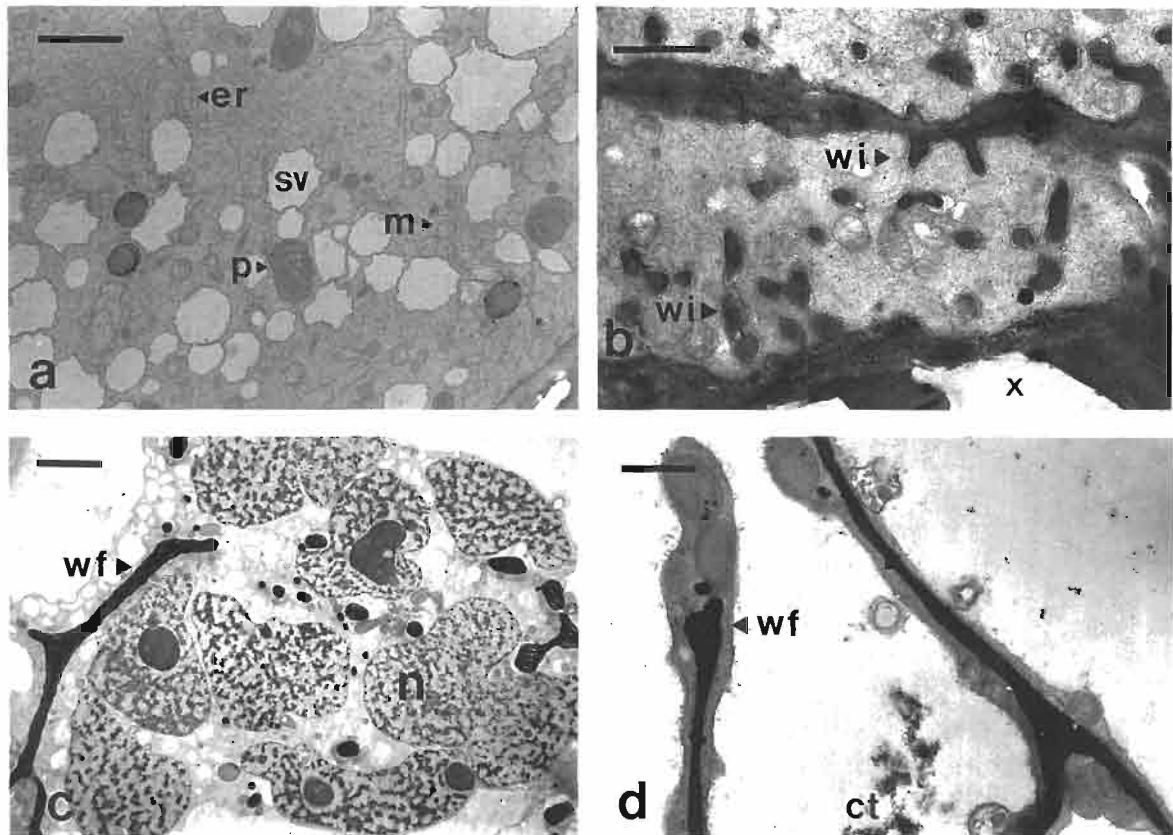
Fig. 4. Ultrastructure of syncytium in susceptible and resistant wheat cultivars – Susceptible cultivar. A : Cytoplasm of day fifteen syncytium with numerous organelles, small vacuoles and abundant endoplasmic reticulum; B : Membrane-bound wall-ingrowths adjacent to xylem in day nineteen syncytium – Resistant cultivar; C : Cluster of hypertrophied amoeboid nuclei with prominent nucleoli and scattered chromatin in day seventeen syncytium, surrounded by small quantity of cytoplasm with few organelles; D : Wall fragments with thin layer of cytoplasm containing plastids and mitochondria. Free chromatin released from degenerated nucleus. er = endoplasmic reticulum, sv = small vacuole, p = plastid, m = mitochondria, wi = wall ingrowth, wf = wall fragment, ct = chromatin, x = xylem. *(Bar equivalents : A, B, D = 2 μm; C = μm.)*

The resistant response mediated by the AUS 10894 CCN resistance gene is evidenced by differences in syncytia of the resistant cultivar at about fifteen days. Syncytia became largely vacuolate, cytoplasm was very reduced and membrane degeneration was observed. Nutrient flow to the nematode may therefore be reduced, at a stage when the female demand for nutrients is high. This type of resistant response, where syncytia are initiated but break down at some later stage, is common with *Heterodera* species and with some other nematode genera. Syncytia induced on resistant *Rhaphanus* by *H. schachtii* show an increase in vacuolation three days after formation and cytoplasm was very compressed with tonoplast breakdown after five days (Wyss *et al.,* 1984). A similar response occurred in potato cultivars resistant to *Globodera rostochiensis* (Rice *et al.,* 1985), although different resistance genes produce varying responses (Rice *et al.,* 1987). In contrast, the resist-

ant response to *Meloidogyne* spp. is usually a quick hypersensitive reaction of necrosis surrounding the syncytium (Paulson & Webster, 1970).

Reports on the structure of syncytia in the resistant wheat cultivar AUS 10894 found that late initiation of syncytia (Grymaszewska & Golinowski, 1991), increased necrosis and early syncytial degeneration (Grymasezwska & Golinowski, 1987) were signs of a host resistant reaction. Cysts were used as inoculum, rather than the quantitative synchronous inoculation system reported here. O'Brien and Fisher (1974) showed that when AUS 10894 is challenged with the Australian pathotype of *H. avenae,* an equal number of juveniles penetrates the roots and initiates galls in both the resistant cultivar and a susceptible cultivar. This report finds the same for the AUS 10894 × Prins cross, with syncytia being established by four days. The necrotic reaction was similar in both the susceptible and resistant culti-

vars, and syncytial differences became apparent just prior to the third moult of the juvenile, which occurs at seventeen days when plants are grown at 15 °C (Fisher, 1981).

A major feature of the syncytial breakdown of the resistant response noted in this study and by others, is that male development is unaffected, either because of their lower nutrient requirement or because they finish feeding before the syncytium degenerates. While the timing of the breakdown differs between species, its occurrence at a stage which does not affect males may indicate that it is the female that triggers the response at a particular point in its development. *H. avenae* juveniles initially increase body size slowly at 15 °C, then female body size increases rapidly after males have stopped feeding. A gene product produced by the female at this stage may induce the host resistance response. This hypothesis fits with the accepted model of gene-for-gene interactions inducing host resistance reactions, and would explain the observed delay in cellular responses to the pathogen.

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