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Morphological and Histochemical Changes Occurring during the Life-span of Root-tip Galls on *Lolium perenne* Induced by *Longidorus elongatus*[1]

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Abstract: The RNA and protein content of perennial ryegrass root-tip galls induced by *Longidorus elongatus* were measured from transverse sections and the morphology described. Galls progressed through five distinct stages and were viable for only 10–12 days at 18 C, after which they collapsed and became necrotic. In the initial stage hypertrophy occurred and cells contained enlarged nuclei and nucleoli, a greater proportion of cytoplasm, and increased concentrations of protein. This was followed by hyperplasia; cells divided to give two or four daughter cells, accompanied by a proportionate reduction in volumes of cytoplasm, nuclei, and nucleoli and reduced concentrations of RNA and protein. The third stage was secondary hypertrophy with enlarged, amoeboid nuclei and nucleoli and a significant increase in concentration of RNA and protein. In the final two stages, as feeding by *L. elongatus* progressively removed cell contents, most cells were devoid of inclusions and galls collapsed and were invaded by soil bacteria. This ordered development and exploitation of galls suggests that *L. elongatus* may have two phases in its feeding.

Key words: morphology, stereology, RNA, protein, host–parasite relationship.

Longidorus elongatus (16,26) forms root-tip galls on strawberry (*Fragaria vesca*) by hypertrophy and hyperplasia (31), but Wyss (32) reported that in celery (*Apium graveolens*) the galls contained only hypertrophied cells or necrotic cells with degraded nuclei. However, galls on celery induced by *L. apulus* are formed by hypertrophy and hyperplasia; some cells are binucleate and others contain nuclei in a state of dissolution (1–3). Galls induced by *L. africanus* on bur marigold are formed by hyperplasia only (4) and contain increased amounts of DNA, RNA, and protein compared to healthy root-tips (6,7). A preliminary study of the histological changes occurring in perennial ryegrass galls induced by *L. elongatus* indicated that root-tip swelling was due to hypertrophy and that nuclei were enlarged with amoeboid outlines, although in older galls only epidermal nuclei were present (19). The changes in morphology, RNA, and protein content of ryegrass galls induced by *L. elongatus* were followed to determine whether hypertrophy or hyperplasia occur and to observe the effect of nematode feeding on host plant nuclei.

Materials and Methods

Small plastic pots (25-ml capacity) were filled with a 1:1 mixture of steam sterilized, air-dried loam and sand with a particle size between 150 and 1,410 μm. The pots were inoculated with 20–40 large, mostly adult *L. elongatus*, planted with two 3-day-old ryegrass seedlings (*Lolium perenne* L. cv. S24), and randomly positioned in a constant temperature cabinet maintained at 19 ± 1 C (24). Five main root-tip galls, together with five control root-tips from noninoculated pots, were collected 2, 4, 8, 10, and 12 days after inoculation. After 10 days two types of gall were found—non-necrotic galls (designated 'young,' 10 days) and galls containing many necrotic cells (designated 'old,' 10 days). The results from the two types of gall are presented separately.

The galls and control root-tips were fixed at 20 C in 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.0–7.5, 350–400 mOsm kg^{-1}) for 20 minutes under partial vacuum and rinsed in 0.05 M phosphate buffer containing sucrose (pH 7.0–7.5, 350–400 mOsm kg^{-1}) for 2 days at 0–4 C. Before being dehydrated each specimen was set in a small block of 1% water agar and embedded in a glycol methacrylate (2-hydroxyethyl methacrylate) monomer mixture (8), made using glycol methacry-

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late purified by the method of Frater (9). The resin was polymerized at 45 C for 3 days and transverse sections (ca. 4 μm thick) were cut using tungsten coated glass knives (18). Serial sections were placed alternately on separate slides, stretched on a drop of 10% ethanol, and dried at 45 C. The thickness of each section was determined by double beam interferometry (20) and the area of each section measured with the aid of a digitizing tablet (10).

Sections were stained either for RNA using Azure B (22) or for protein using Naphthol Yellow S (23). Amounts of RNA or protein in each section was determined with a Vickers M85 scanning microdensitometer set as follows: wavelength 470 nm (Azure B) or 590 nm (Naphthol Yellow S); spot diameter 1 μm; bandwidth 70 nm; threshold 0.05; and a 400-μm-d mask. Results were calibrated against biochemically determined RNA (5,14) and protein (15) contents of 40 control root-tips. Total RNA and protein contents of each gall or root-tip were calculated from the section area and thickness measurements.

Volume fractions of cytoplasm, nucleus, nucleolus, cell wall, vacuole, and intercellular space, as well as the surface area of cell wall, nucleus, nucleolus, and vacuole, were determined by stereology (11) using a Weibel type 2 lattice on an eyepiece graticule in a light microscope at ×1,250 magnification. A preliminary test showed that the application of 400 points over each root-tip would give the volume fraction of nucleus to within 5%. The lattice was then applied in a systematic pattern from a random start (29) until at least 600 points had been laid over each root-tip. The shape factors of cells, nuclei, nucleoli, and vacuole were calculated according to Osterby and Gunderson (17).

Results

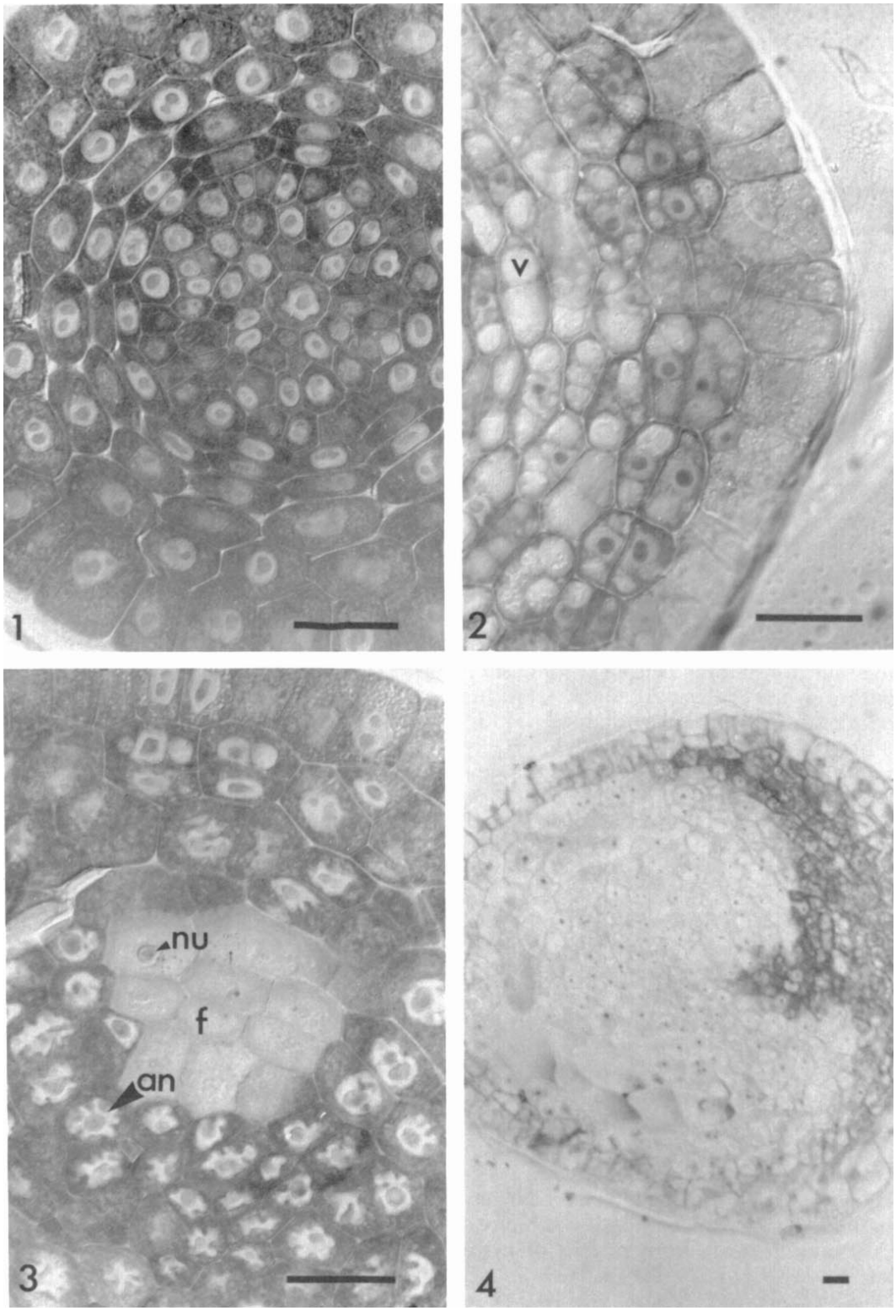
Morphological changes: Root-tips collected 2 days after inoculation were not noticeably swollen but contained small, necrotic feeding sites in the meristem. Older galls were swollen in the meristem region and had diameters 1.5–2.5 times greater than control root-tips. After 10 and 12 days many galls contained extensive necrotic areas or had collapsed. Morphological changes induced by *L. elongatus* could be classified in distinct stages which did not entirely correspond with the supposed age of the gall. Therefore, the changes are described according to these stages because this gives a clearer understanding of the processes occurring within galls induced by *L. elongatus.*

Control root-tips: Nondividing cells in control root-tips contained a single spherical nucleus with 1–4 spherical nucleoli (Fig. 1). Epidermal cells, the largest cell type, contained cytoplasm which stained lightly with Azure B and many small and medium vacuoles. A few cells had lobed nucleoli. Some epidermal cells had lignified walls, as indicated by a turquoise color (12). Outer cortical cells resembled the epidermal cells, but inner cortical and procambial cells had dense (i.e., no vacuoles), heavily stained cytoplasm and ovoid nuclei.

Stage 1—initial hypertrophy: After 2 days root-tips fed upon by *L. elongatus* had enlarged cells, nuclei, and nucleoli. Some nuclei had a multi-lobed outline and several contained an increased number of nucleoli, while some nucleoli in cortical cells contained clear areas. The large central vacuole, characteristic of control epidermal and outer cortical cells, had been replaced by numerous smaller vacuoles in many cells. Sites of nematode feeding were identified by the presence of necrotic cells (collapsed cells containing a dense, granular staining reaction with Azure B) or empty cells devoid of cytoplasm which maintained their shape, but no cellular changes occurred preferentially around such feeding sites.

Stage 2—hyperplasia: Some galls collected 4 and 8 days after inoculation showed organized hyperplasia and synchronized division of the cortical and procambial cells (Fig. 2). Cells had divided to give two or four uninucleate daughter cells. All cells contained large vacuoles, but the degree of vacuolation was not uniform and tended to be greater on one side of the root than on the other. Size of nuclei and nucleoli was markedly reduced compared with those in stage 1, and some nuclei were adpressed to the plasmalemma or between vacuoles. The cytoplasm of some cortical cells was heavily stained with Azure B, and there were squashed necrotic cells in some sections. Soil bacteria, appearing as purple staining rods, were present on the outside of necrotic epidermal cells.

Stage 3—secondary hypertrophy: Galls taken 4, 8, and 10 days after inoculation showed general cell enlargement and an



FIGS. 1–4. Root tip galls on *Lolium perenne* induced by *Longidorus elongatus*. Transverse sections. Scale bar for figures = 50 μm. 1) Control ryegrass root tip. 2) Stage 2 gall showing hyperplastic division of cortical cells and large vacuoles (v). 3) Stage 3 gall with a feeding site (f), containing a nucleolus (nu) and surrounded by cells having nuclei with an amoeboid outline (an). 4) Stage 4 gall showing the removal of cytoplasm from large areas of the root.

TABLE 1. Volume ($mm^3 \times 10^{-3}$) of cell components and proportion (as percentage of root-tip volume) in root tips fed on by *Longidorus elongatus*.

Cell component	Control		Stage of gall development*								LSD†
			1		2		3		4		
	Vol.	%	Vol.	%	Vol.	%	Vol.	%	Vol.	%	Vol.
Cytoplasm	91	66	127	72	77	39	216	68	92	24	116
Nucleus	14	10	17	9	16	8	33	11	18	5	16
Nucleolus	4	3	7	4	2	1	7	2	2	1	3
Vacuole	19	14	14	8	87	44	41	13	214	56	62
Cell wall	7	5	9	5	8	4	10	3	9	2	N.S.
Other‡	3	2	3	2	8	4	9	3	44	12	N.S.
Root-tip	138		177		197		315		378		140

* Stage 5 galls which are mostly empty cells were omitted.
† Least significant difference ($P < 0.05$) calculated using 20 degrees of freedom.
‡ Includes empty and necrotic cells and intercellular space.

increase in the amount of cytoplasm in cells. In some sections a group of inner cortical and procambial cells was devoid of stain. These cells were a uniform light brown and contained no organelles apart from the occasional degraded nucleus which still contained a relatively large nucleolus. Some of the other nonstaining cells in feeding sites contained several small, dark staining granules, while others contained faintly staining patches of cytoplasm. The cells surrounding the area where cell contents had been removed contained amoeboid nuclei with diffuse, amoeboid nucleoli and dense, heavily stained cytoplasm (Fig. 3). Others contained a diffuse nucleus, and some nuclei contained faint staining nucleoli. In other cells near the feeding site the nuclei had taken on elaborate amoeboid outlines. Cells further away from the feeding site all contained discrete nuclei, some of which were amoeboid. There was, however, always a clear demarcation between the empty cells in the feeding site and adjacent cytoplasm enriched cells. Cells further away from the feeding area were enlarged, with less heavily stained cytoplasm, and contained nuclei with more regular outlines. Epidermal cells were largely unaffected, even in sections in which more than half the cells were devoid of cytoplasm.

Stage 4—ingestion of cell contents: Between 8 and 10 days after inoculation, epidermal cells opposite the feeding site had started to divide and in some sections were the only cell type to contain cytoplasm, all other cell types being entirely devoid of cytoplasm or organelles (Fig. 4). In some sections squashed, necrotic epidermal cells were seen, immediately below which was an accumulation of cytoplasm in the outer cortical cells. No cell walls were observed where cytoplasm accumulated, but all other cell walls were present and there were no signs of cell wall breakdown. Some empty cells in the center of the root contained prominent nucleoli. Many soil bacteria occurred around the root and inside some empty epidermal cells.

Stage 5—collapse of the gall: The final stage was present in the necrotic galls taken 10 and 12 days after inoculation. Most cortical and procambial cells were completely empty with intact cell walls, and there were many necrotic cells. A cavity had developed in the center of some roots, the result of cells collapsing rather than cell wall breakdown, and contained many Azure B positive rods that were probably actinomycetes.

Stereology: As seen in Table 1, the amounts of cytoplasm, nucleus, and nucleolus increase, in relation to total volume, during hypertrophy (stages 1 and 3) and decrease during hyperplasia (stage 2) and ingestion (stage 4), whereas the amount of vacuole decreases during hypertrophy and increases during hyperplasia and ingestion. The amount of cell wall remains constant. During gall formation the total volume of the root-tip gradually increases,

TABLE 2. Surface areas of all components in root-tips of ryegrass fed on by *L. elongatus* expressed (a) per unit volume ([$mm^2 \times mm^{-3}$] $\times 10^{-4}$) and (b) in total ($mm^2 \times 10^{-4}$).

Cell component	Control		Stage of gall development*								LSD†	
			1		2		3		4			
	a	b	a	b	a	b	a	b	a	b	a	b
Cell wall	347	47	316	56	328	61	312	96	263	99	42	39
Nucleus	157	22	90	16	78	15	116	36	43	18	76	18
Nucleolus	43	6	53	9	24	5	42	12	14	5	12	4
Vacuole	126	18	84	16	271	52	127	34	247	92	121	30

* Stage 5 galls which contain mostly empty cells have been omitted.
† Least significant difference ($P < 0.05$) calculated using 20 degrees of freedom.

but the total volume of each cell component can fluctuate significantly depending on the proportion of each component.

The calculated surface area per unit volume and the calculated total surface area of nucleus and nucleolus within a gall also tend to increase during hypertrophy and decrease during hyperplasia and ingestion (Table 2). The values for cell wall and vacuole decrease during hypertrophy and increase during hyperplasia and ingestion. The shape factors of cell components were calculated to establish if widespread changes could be detected in the early stages of gall development. No significant changes in shape factor were noted until stage 4, when cells, nuclei, and vacuoles were significantly less spherical than in control roots.

RNA and protein content: Significant decreases in the concentration of RNA and protein occurred during stages 2 (hyperplasia) and 4 (ingestion) and, although no change was detected during stage 1 (initial hypertrophy), a significant increase was seen during stage 3 (secondary hypertrophy) (Table 3). The total amounts of RNA and protein in a gall show a significant increase during stage 3 and a decrease during stage 4 (ingestion).

DISCUSSION

The data presented indicate that ryegrass galls induced by *L. elongatus* progress through distinct stages of development. Initially there is hypertrophy causing an increase in the size of cells, nuclei, and nucleoli, an increase in the proportion of cytoplasm, and a slight increase in the concentration of protein. This is followed by hyperplasia, cells dividing to give two and four daughter cells, which is accompanied by an apparent reduction in the proportions of cytoplasm, nucleus, and nucleolus and a large increase in the proportion of vacuole. The reduced concentration and total amounts of RNA and protein could be due partly to utilization during cell division and partly to removal during nematode feeding. Secondary hypertrophy then follows, and the increased amount of cytoplasm and enlarged amoeboid nuclei and nucleoli, together with the large increase in concentration of RNA and protein, all suggest that RNA and protein synthesis are enhanced. The most extensive removal of cell contents by *L. elongatus* occurs during secondary hypertrophy. Feeding zones, indicated by areas within the gall where the cells are devoid of cytoplasm, are initially small but become progressively larger until almost all the cells are largely empty. During stages 3 to 5, cytoplasm accumulates in some epidermal and outer cortical cells and the gall finally collapses after the contents have been removed from almost every cell in the gall.

Hypertrophy and hyperplasia frequently occur in galls induced by *Longidorus* spp. (1–4,31,32). Hyperplasia induced by *L. apulus* in celery, however, was disordered and led to abnormal tissue proliferation (2), whereas the hyperplasia induced by *L. elongatus* in ryegrass was highly ordered. No cell wall ingrowths were seen in the course of this study, as have been observed in galls on celery induced by *L. apulus* (3), but their formation may depend on the host status of the plant. The dissolution of celery nuclei caused by *L. apulus* (3) could

TABLE 3. The concentration ($\mu g\ mm^{-3} \times 10^{-1}$) and total RNA and protein content ($\mu g \times 10^{-1}$) in root-tip galls of ryegrass induced by *Longidorus elongatus*.

		Stage of gall development					LSD*	
	Control	1	2	3	4	5	5%	1%
RNA								
Concentration	1.22	1.00	0.62	0.82	0.22	0.25	0.16	
Total content	0.17	0.18	0.12	0.26	0.08	0.06		0.13
Protein								
Concentration	37.92	36.96	23.10	29.57	8.32	9.24	4.54	
Total content	5.31	6.65	4.62	9.46	3.16	2.31		4.25

* Least significant difference calculated with 60 degrees of freedom.

be the process by which the nuclei become disrupted within feeding zones in *L. elongatus* galls of ryegrass. Wyss (32) noted the presence of nucleoli within necrotic cells in *L. elongatus* galls on celery, and nucleoli were often the only organelle present within feeding zones in *L. elongatus* galls of ryegrass.

The early cellular changes induced by *L. elongatus,* before extensive removal of cellular contents occurs, are similar to those induced by *Xiphinema* spp. (19,21,30,33) and *Meloidogyne* spp. (13). Galls induced by these genera contain hypertrophied cells with enlarged nuclei having amoeboid outlines, enlarged nucleoli, and dense cytoplasm. Multinucleate cells arise in galls induced by *X. index* (33) and *Meloidogyne* spp. (13) from mitosis without cytokinesis, and multinucleate cells presumably do not develop in galls induced by *L. elongatus* because mitosis and cytokinesis occur together during hyperplasia. The fact that the stereological data do not clearly reflect the large changes in, for example, nuclear size and shape in some cells (see Fig. 3) is probably due to the localized nature of some cell modifications. Although modifications induced by *L. elongatus* may be more widespread than those induced by *Xiphinema* spp., the large proportion of less modified cells within a gall is reflected by the small changes in the stereological results. Modifications such as the increased concentration of RNA and protein, the larger size of nucleoli, and the invaginated profiles of the nuclei all suggest that host metabolism is enhanced during secondary hypertrophy. These changes result in root-tip galls becoming a better food source than non-modified root-tips.

The effect of *L. elongatus* on root-tip galls of ryegrass during the removal of the cell contents is markedly different from the processes described for galls induced by other nematodes. During feeding *L. elongatus* inserts its stylet deep into the root-tip and it often remains in the same cell for several hours, an initial inactive period being followed by a period of ingestion (20). The removal of cell contents in cells adjacent to that penetrated by the stylet tip could be brought about by enzymes in the dorsal gland cell secretions injected during the initial period of inactivity. A similar pattern of behavior was described for *L. caespiticola,* which also remained with its stylet in the same cell for up to 60 minutes before ingestion began, during which time it was thought to be injecting saliva (27). This long delay before ingestion commences could account for the widespread cellular modifications induced by *Longidorus* spp. and also enable the spread of virus particles through the root-tip, as these are thought to be released from the sites of retention and inoculated into the plant while the nematode is injecting saliva (25).

The ordered development of *L. elongatus* galls points to there being a period during which cellular modification is initiated and abstraction is small followed by a period during which cell contents are mobilized and removed. It could be that *L. elongatus* has two types of feeding behavior, one to initiate gall formation and another to remove cell contents, or alternatively that cell contents are only removed after prolonged feeding. In both instances it is likely that cell components are broken down into smaller molecular fractions which can be drawn to the stylet and ingested. Such a movement towards the stylet could explain the accumulation of cytoplasm in older galls

and agrees with previous suggestions that food is derived from cells far removed from the cell which contains the stylet tip (27,28).

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