# Development of Galls Induced in *Lolium rigidum* by *Anguina agrostis*

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## ABSTRACT

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The growth and development of galls induced by *Anguina agrostis* were observed in the inflorescences of annual ryegrass (*Lolium rigidum*) plants harvested at weekly intervals from inoculated field plots. Infective dauer larvae of *A. agrostis* colonized plants during their vegetative growth phase, and gall induction had occurred by the time floret development was readily discernible within developing spikelets. Galls typically developed in place of ovules, less commonly in place of stamens, and rarely on glumes or rachis. The galls, which grew rapidly as nematodes developed, consisted of a wall several cell layers deep surrounding a central cavity. Cells adjacent to the cavity were modified, presumably to provide metabolites for development and reproduction of the nematodes, which completed a single generation before plant senescence occurred and the galls dried out. Cells at the surface were unchanged, thus maintaining the structure of the galls.

*Additional key words*: annual ryegrass toxicity, *Corynebacterium rathayi*, ultrastructure, callose.

The general pattern of development of galls induced in the inflorescences of grasses and cereals by *Anguina agrostis* and *A. tritici*, respectively, has been described by several workers (4,6,10, 15–18,20,21,23). Apart from minor differences reported by these workers, mainly concerning the sequence of infection and the tissues involved in gall formation, this pattern is essentially similar for both species. However, no detailed study has been made of the morphological changes that take place during gall development.

The induction of galls by *A. agrostis* in *Lolium rigidum* Gaudin has particular significance, because the disease known as annual ryegrass toxicity has become widespread in Australia in recent years and is responsible for numerous stock mortalities (1,14,28). In this disease, the galls are not only nematode reproduction sites but may also be colonized by *Corynebacterium rathayi* and subsequently become toxic to grazing animals (19,27). A detailed study of gall development and host response to both organisms should help determine the origin and nature of the toxin and provide a sound basis for the development and improvement of strategies to control the disease.

A comparison of mature galls containing nematodes with those colonized by *C. rathayi* was reported earlier (3). In this article, we describe the morphological development of galls in which the nematodes develop and reproduce.

## MATERIALS AND METHODS

**Source of infected material**. In April 1978, field plots of infected ryegrass were established at Katanning, Western Australia. Single seeds of ryegrass were sown with three galls at 10-cm intervals in four plots 2 × 0.6 m. The galls had been collected from mature ryegrass pasture the previous season and each contained an average of 1,500 infective dauer larvae (L2) of *A. agrostis*. One plant was collected from each plot at intervals of one week from germination in late May until the ryegrass matured in mid-November. The plants were placed in 4% paraformaldehyde in 0.115 M phosphate buffer (pH 7.3) at 5 C and stored at this temperature (26). Shoot apices and developing inflorescences dissected from these plants provided a range of infected material that was further processed for light and electron microscopy. Only galls containing nematodes were examined in this study; those colonized by bacteria were not used.

**Light microscopy**. Shoot apices and galls at various stages of development were dehydrated at 5 C successively with 2-methoxyethanol, ethanol, *n*-propanol, and *n*-butanol and embedded in glycol methacrylate (13). Sections 2 μm thick were cut with an LKB ultramicrotome using glass knives, stained using the periodic acid-Schiff, toluidine blue technique (13), and examined under bright field. Alternatively, sections were stained with 0.5% water-soluble aniline blue in 0.15 M $KH_2PO_4$, pH 8.3, and examined for the presence of callose under ultraviolet light, using a Zeiss filter combination specific for the aniline blue excitation peak (25).

**Electron microscopy**. Fixed material from infected shoot apices and galls was washed in 0.115 M phosphate buffer, fixed in 1% osmium tetroxide in the same buffer for 1 hr at 23 C, washed again in buffer and then distilled water, and finally taken through an ascending ethanol series into araldite, using propylene oxide as an intermediary. Thin sections were cut with glass knives, stained in a saturated solution of uranyl acetate in 50% methanol for 20 min at 23 C, and then placed in lead citrate for 10 min at 23 C. The sections were examined in a Philips EM 400 transmission electron microscope at 80 kV.

## RESULTS

Colonization of the ryegrass plants by infective larvae of *A. agrostis* began in mid-July, 7 wk after germination, and continued for a further 9 wk while the plants were tillering. During this period of vegetative growth, the plants maintained a prostrate habit and larvae migrated toward the main and lateral shoot apices as they successively developed. Most colonization occurred when each shoot apex was protected by either one or two leaves, and the nematodes remained in the vicinity of these apices as other leaves were initiated (Fig. 1).

The infective larvae showed no morphological change and did not appear to modify the plant during floral initiation or in the early stages of differentiation of the spikelet primordia (Figs. 2–4). High numbers aggregated around floret primordia as reproductive development of the plants progressed (Figs. 5 and 6), and

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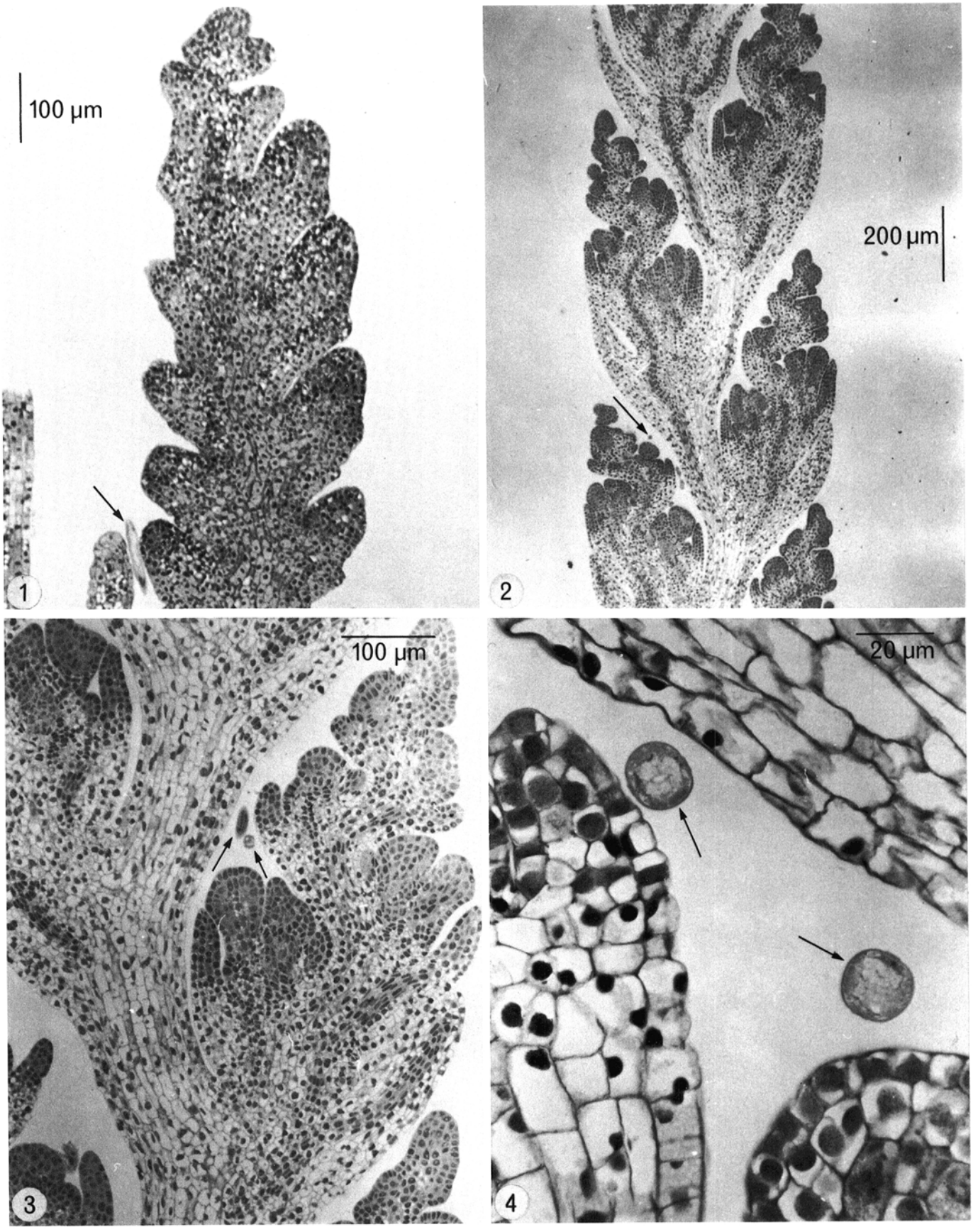
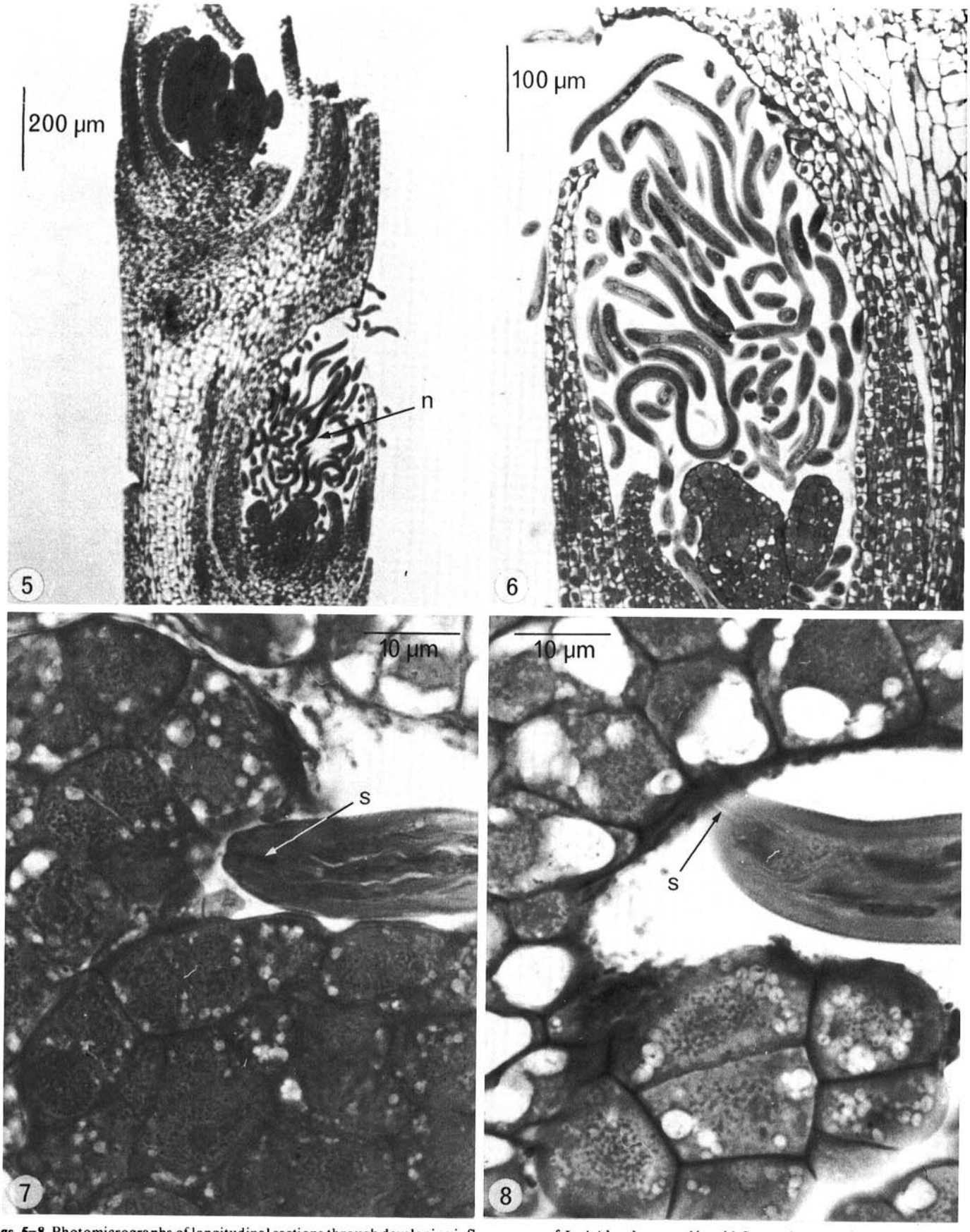
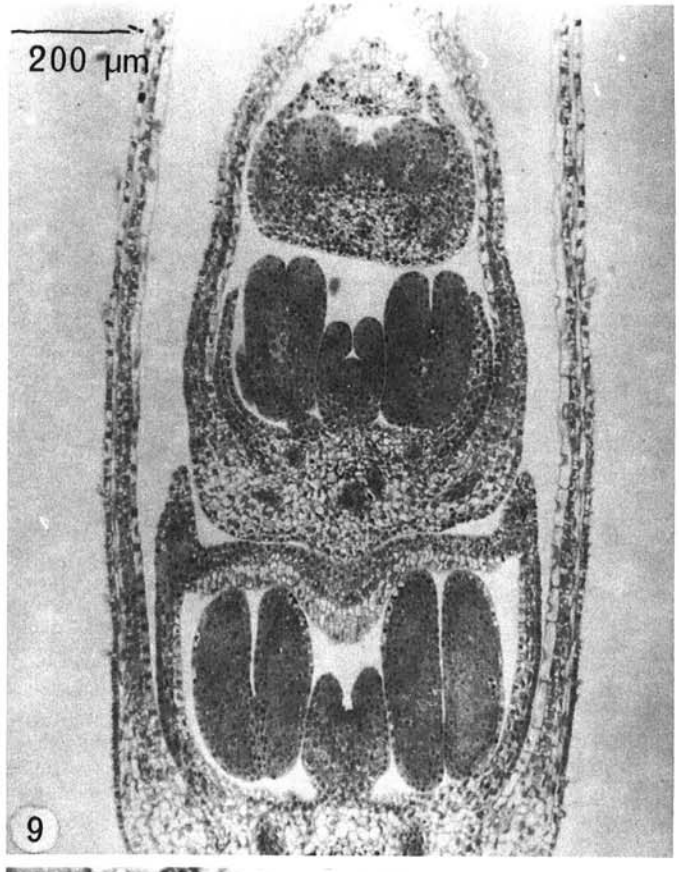
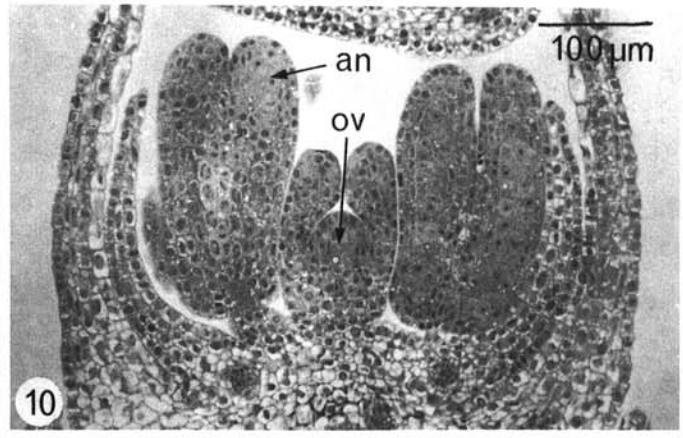


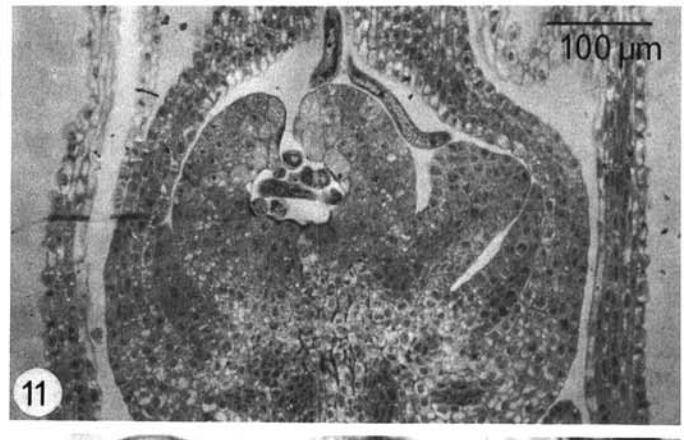

**Figs. 1–4.** Photomicrographs of longitudinal sections through developing inflorescences of *L. rigidum* harvested in mid-September, showing dauer L2 of *A. agrostis* (arrows) that are attracted to the growing points of the spikelet primordia. **1,** Early stage of spikelet primordia development. **2,** Later stage of development. **3,** Higher magnification of stage shown in Fig. 2. **4,** Still higher magnification of stage depicted in Figs. 2 and 3, showing cross sections of two dauer L2s adjacent to meristematic tissue.

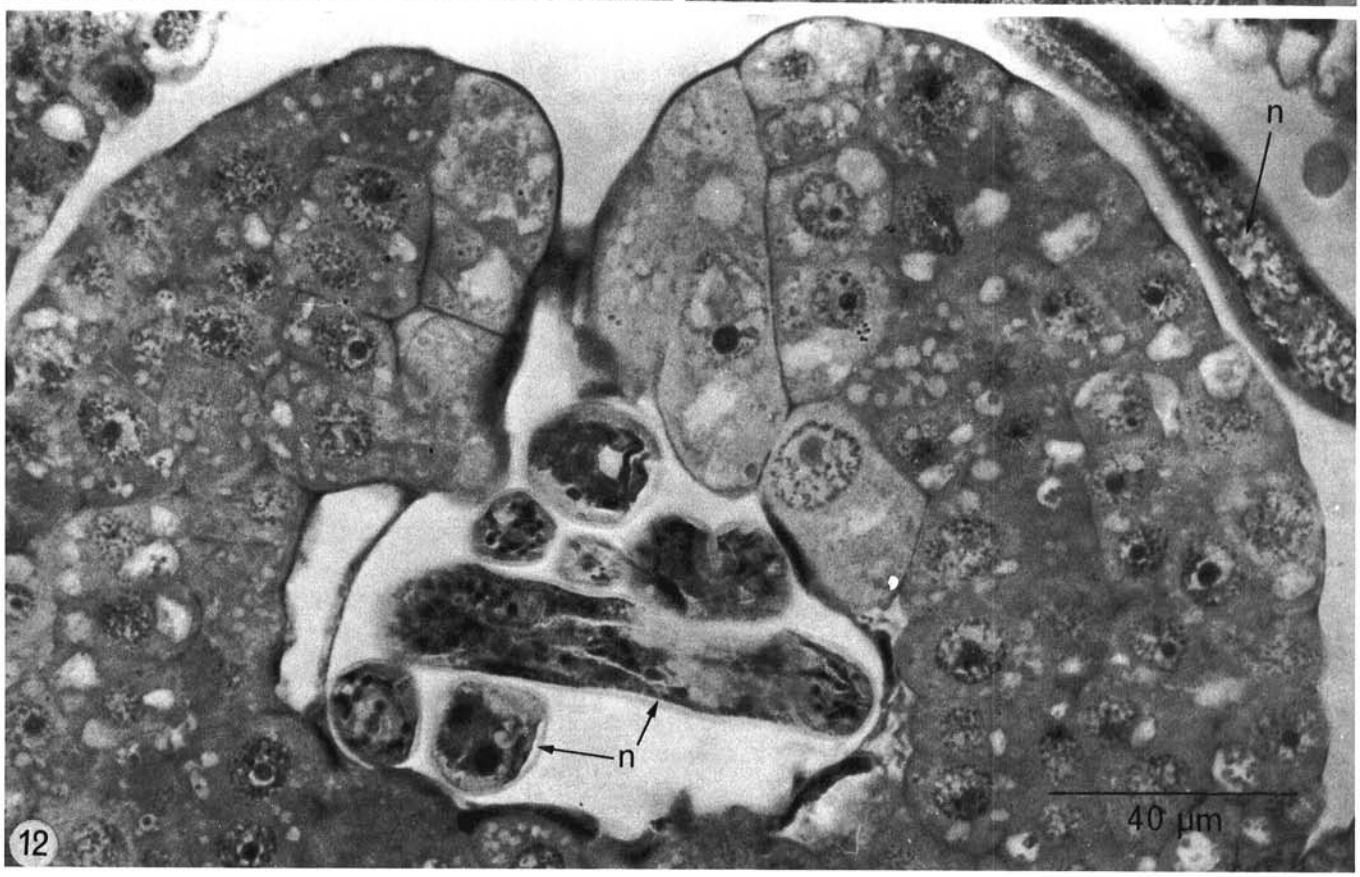



**Figs. 5–8.** Photomicrographs of longitudinal sections through developing inflorescences of *L. rigidum* harvested in mid-September, showing dauer L2 of *A. agrostis* (arrows) feeding. **5,** Large numbers of nematodes (n) clustered around a floret primordium. **6,** Higher magnification of Fig. 5, showing distribution of infective dauer L2 more clearly. **7** and **8,** Higher power photomicrographs of L2 observed under oil immersion, showing position of the buccal stylet (s) during feeding: stylet retracted (Fig. 7) and everted into area adjacent to plant meristematic tissue (Fig. 8).




**Figs. 9–12.** Lower power photomicrograph of a section through a developing spikelet harvested in mid-September, showing three florets of *L. rigidum*. **10** and **11,** Photomicrographs, at the same magnification, of uninfected (Fig. 10) and infected (Fig. 11) florets of *L. rigidum*, with Fig. 10 showing normal development of anthers (an) and ovary (ov). **12,** Photomicrographs at higher magnification of Fig. 11, showing nematode larvae (n) feeding in the young ovarian gall. Cell on the left side is devoid of cytoplasm and densely staining material lines the lumen of the gall.

individual larvae were seen with stylets retracted and protruded, apparently browsing on plant cells or their exudates (Figs. 7 and 8).

Modification of the plants' normal growth habit was first observed in mid-September, 16 wk after germination, when floret development was readily discernible within spikelets (Fig. 9). Differentiation of healthy florets (Fig. 10) was slow by comparison with that of infected florets (Fig. 11), in which nematodes feeding actively at the primordia induced rapid cell division and enlargement. Initially, the cytoplasm of these cells became dense and granular and the nuclei enlarged. Gradually, the cell contents became vacuolated and the cytoplasm depleted; ultimately the cells emptied and collapsed.

This process of general stimulation of growth and simultaneous local destruction of cells initially produced a depression and ultimately a gall comprising a cavity and a distinct opening (Fig. 12). Such galls, which typically developed in place of ovules and less commonly in place of stamens, usually contained three or four nematodes, although some contained as many as nine. Occasionally, two galls developed from a single floret primordium and, in rare cases, galls developed on the glumes and rachis. These rarer instances of galling were seen during this investigation and also in natural field infections in which the level of inoculum was very high.

Once a gall was initiated, the nematodes continued to feed actively, and rapidly molted to adults. During this period of rapid nematode growth and development, a corresponding increase occurred in the density of cytoplasm within cells adjacent to the cavity and, to a diminishing extent, in cells at increasing distance from the cavity. At the same time, the walls of the cells adjacent to the gall cavity became coated with material that stained deeply with toluidine blue (Figs. 13 and 14). This material appeared to originate within the cells and accumulated just before cell breakdown (Fig. 14). In thin sections viewed under the electron microscope, an electron-dense material was seen to have similar distribution (Fig. 15). Higher magnification (Fig. 16) showed that the walls of the collapsed cells containing this electron-dense material initially remained intact, and the adjacent cells were densely packed with organelles indicative of high metabolic activity. The electron-dense material appeared fibrillar in nature at high resolution (Figs. 17 and 18).

By early October, 19 wk after plant germination, the galls ranged in size from 1 to 4 mm and contained adults. As the galls aged, the males remained active, whereas the females became considerably distended by developing ovaries and virtually immobile except in the head region. The younger galls (Fig. 19) usually contained immature adults, and the cells of the gall walls appeared to be metabolically very active (Fig. 20). Some eggs in early stages of development were present in the older galls (Fig. 21), and the cells of these gall walls similarly appeared to be very active. The gall wall was made up of approximately five or six layers of cells, with cells of the outermost layers becoming elongated (Fig. 22). Directional growth of the galls was associated with increasing elongation of the older outermost cells, particularly towards the apex, and proliferation of cells from an actively meristematic region at the base. Within a further 2 wk, most galls were 2.5–4.5 mm long and filled with eggs at various stages of embryonic development (2). In the younger of these galls (Fig. 23), the adults were still actively laying eggs (Fig. 24), whereas in the more advanced galls (Fig. 25), they had died and had begun to break down (Fig. 26). Some eggs had completed embryogenesis, and second-stage larvae were beginning to hatch.

By early November, 22 wk after plant germination, the galls had reached a maximum size of 3–5 mm (Fig. 27) and had become purplish due to the development of anthocyanin pigmentation. All the second-stage larvae had hatched and completed their development to infective dauer larvae. This growth presumably resulted from active feeding by the larvae on plant cells adjacent to the gall cavity, which had almost completely lost their cytoplasmic contents (Fig. 28). Remnants of the adults became increasingly difficult to find. With the onset of dry summer conditions and senescence of the ryegrass, the galls gradually dried out. As this occurred, the larvae became closely appressed against each other (Figs. 28 and 29), and the cells of the gall walls became devoid of cytoplasm and collapsed (Figs. 28 and 30). Histochemical tests showed that callose developed in plant cells lining the cavity of the gall in close proximity to feeding nematodes (Fig. 31).

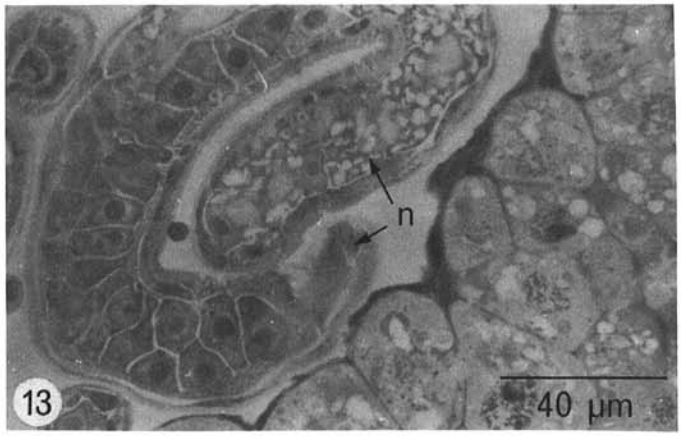
## DISCUSSION

*A. agrostis*, thought to be a recent introduction (2), has successfully adapted to the Australian climate and completes its life cycle synchronously with the development of its host, ryegrass. The anhydrobiotic dauer larvae oversummer in dry galls, emerging in winter to infect ryegrass during the plants' vegetative growth phase. Reproductive development of the plant and nematode proceed simultaneously, although initially the galls develop at a more rapid rate than corresponding uninfected floral tissue. The nematodes complete their development, reproduce, and give rise to a new generation of second-stage larvae, which develop to dauer larvae and become anhydrobiotic as the plants approach senescence in late spring.

As Price et al (23) reported, galls in ryegrass typically develop from floret primordia between growing lemmas and paleas; whereas development of other floral tissue is normally suppressed, this is not always the case. In some instances, single or multiple galls arise from other tissue, including stamens, glumes, and rachis, and the suppression of development of lodicules and stamens is not always complete. In ryegrass, at least, the varying sites of galling probably relate to the density of infecting larvae, and although ovarian tissue is the preferred site, other tissues can become involved as competition for feeding sites increases. We found that gall formation on ryegrass was more variable than that reported for *A. agrostis* on bentgrass (16,18) or for *A. agropyronifloris* on western wheat grass (22) but resembled most closely that for *A. tritici* on wheat (10). However, these differences may simply reflect the influence of the specific range of climatic conditions prevailing when our observations were made.

The structure of the developing galls appeared to be similar to that of galls induced by *A. agrostis* on other hosts, by *A. agropyronifloris* on western wheat grass, and by *A. tritici* on wheat. They consisted of an active meristematic zone at the base and a wall several cell layers deep surrounding a cavity. Cells adjacent to the cavity, and to a lesser extent those further away, were larger, had enlarged nuclei, and were densely packed with organelles presumably indicative of high metabolic activity. Unlike nematodes in the Heteroderidae, which produce very distinctive changes in a relatively small number of cells (12), species of *Anguina* appear to induce more subtle changes in a large number of cells. Nevertheless, the synthesis of metabolites by these altered cells must be greatly increased to support the growth of the invading larvae to adulthood and the eventual development of the next generation of anhydrobiotic dauer larvae. The contents of the cells altered by the nematodes are eventually replaced by dense

**Figs. 13–18.** Photomicrographs of sections through material harvested in the field in late September. This material contained young adults in the process of feeding and growing. **13,** Part of a nematode (n) and the walls of the gall cavity coated with material that stains with toluidine blue. **14,** Parts of nematodes and cell wall breakdown (cwb) in some of the cells lining the gall cavity. **15,** Low power electron micrograph of material similar to that depicted in Figs. 13 and 14. Nematodes are close to cells lining the gall cavity, the cell is almost devoid of cytoplasm in the center, and electron-dense material (ed) is between walls of collapsed cells. Other cells lining the gall cavity are highly vacuolated. **16,** Electron micrograph at higher power than Fig. 15, showing that cell walls (cw) of a collapsed cell lining the gall cavity remain intact and that the cell is full of electron-dense material. The three adjacent cells all have dense cytoplasm containing organelles, indicative of high metabolic activity. Many contain an enlarged nucleus (Fig. 15). **17** and **18,** Higher powered electron micrographs, showing (Fig. 17) relationship between walls of a collapsed cell lining the gall cavity and the enclosed electron-dense material, and (Fig. 18) fibrillar nature of electron-dense material at high resolution.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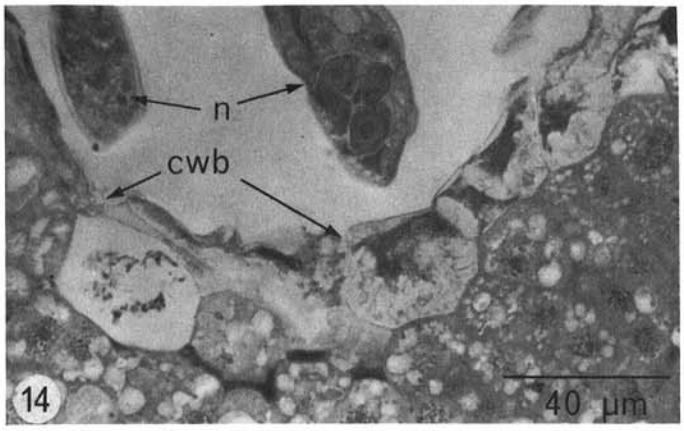
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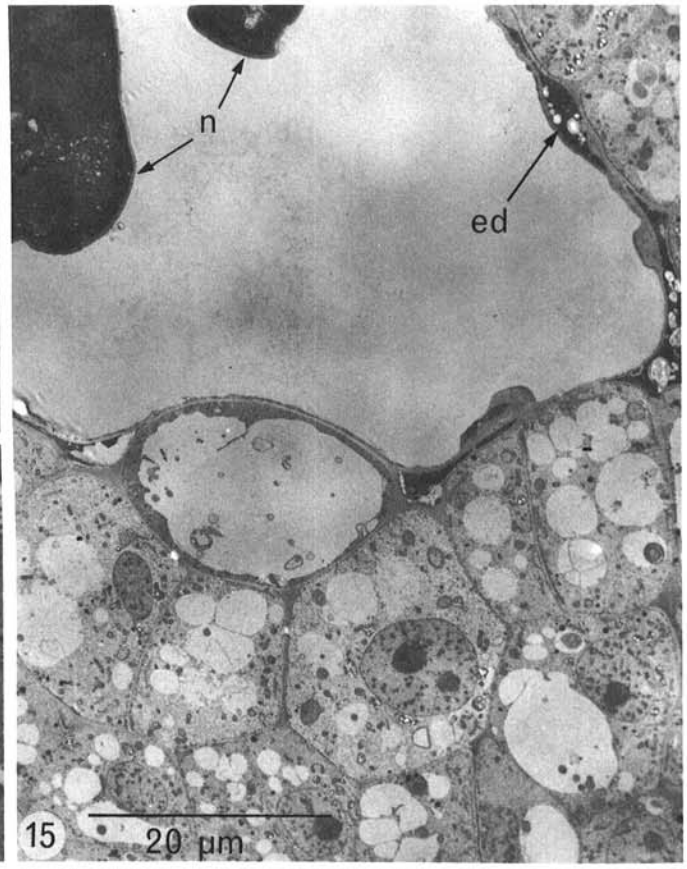
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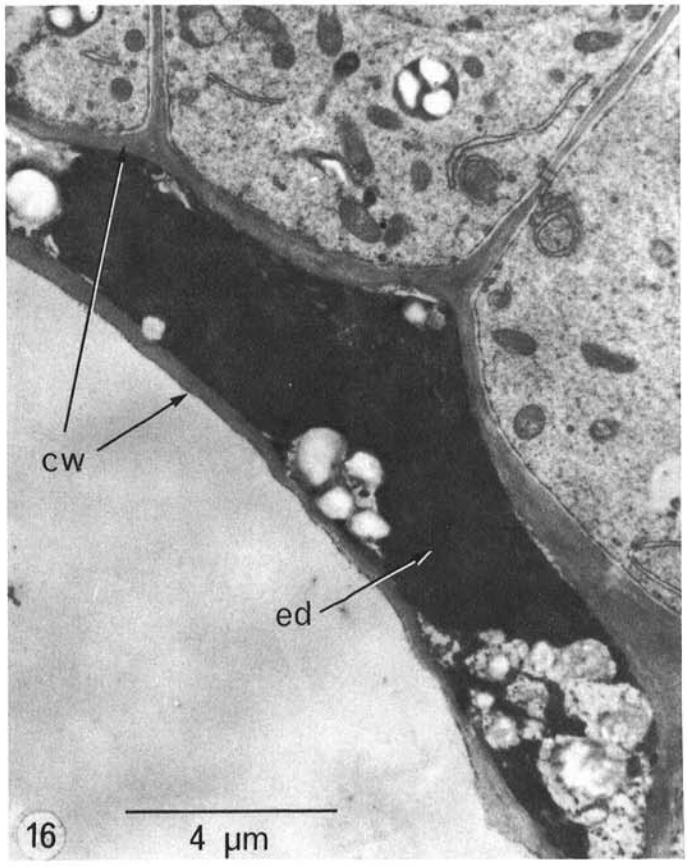
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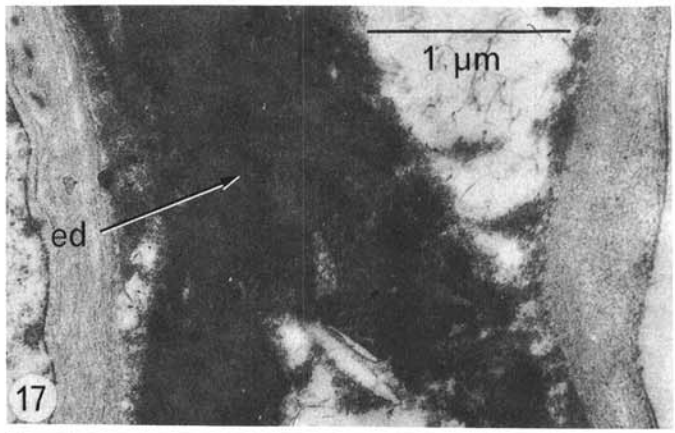
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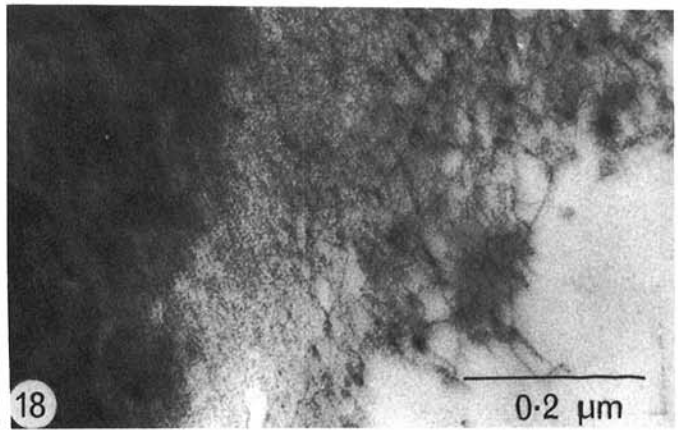
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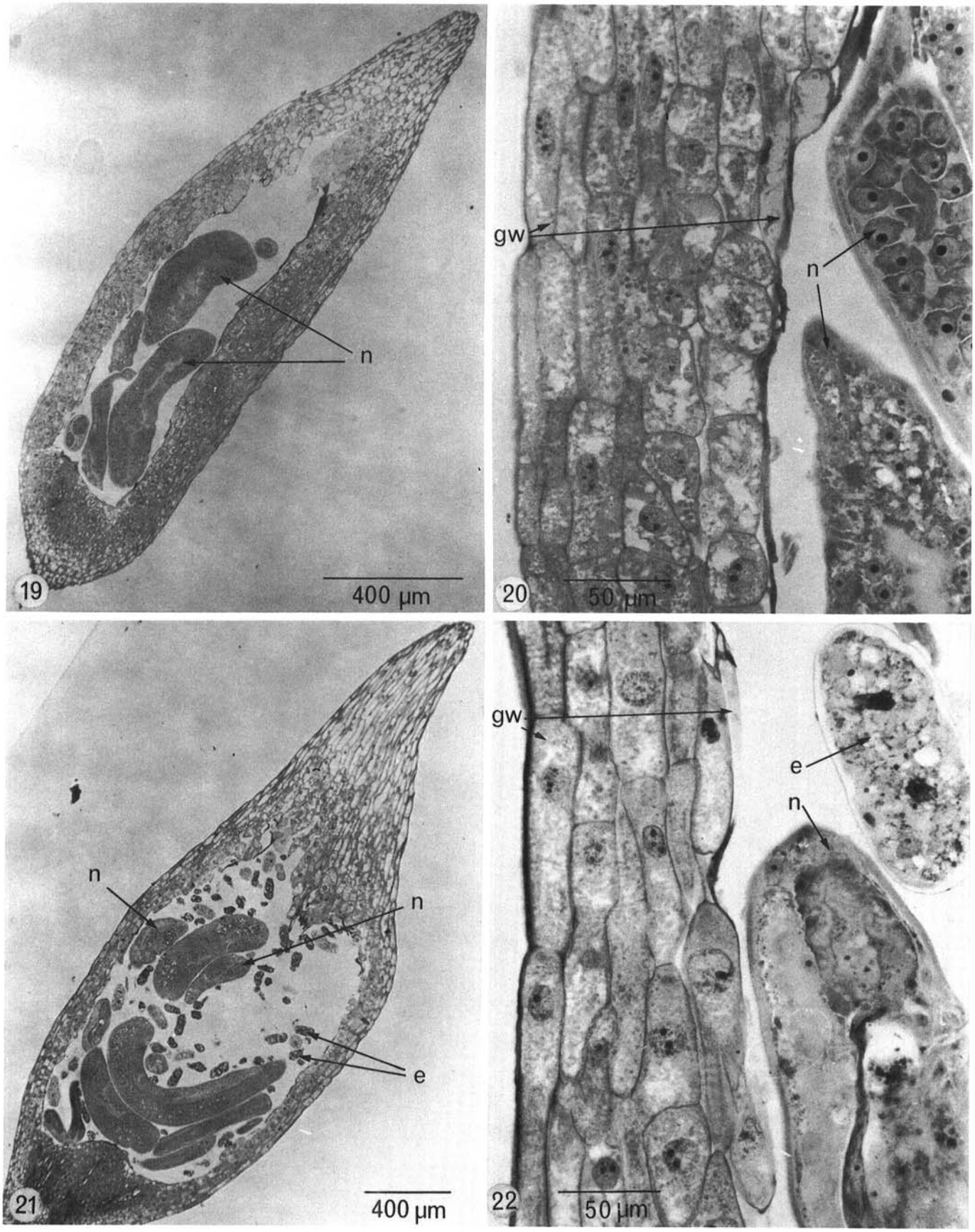
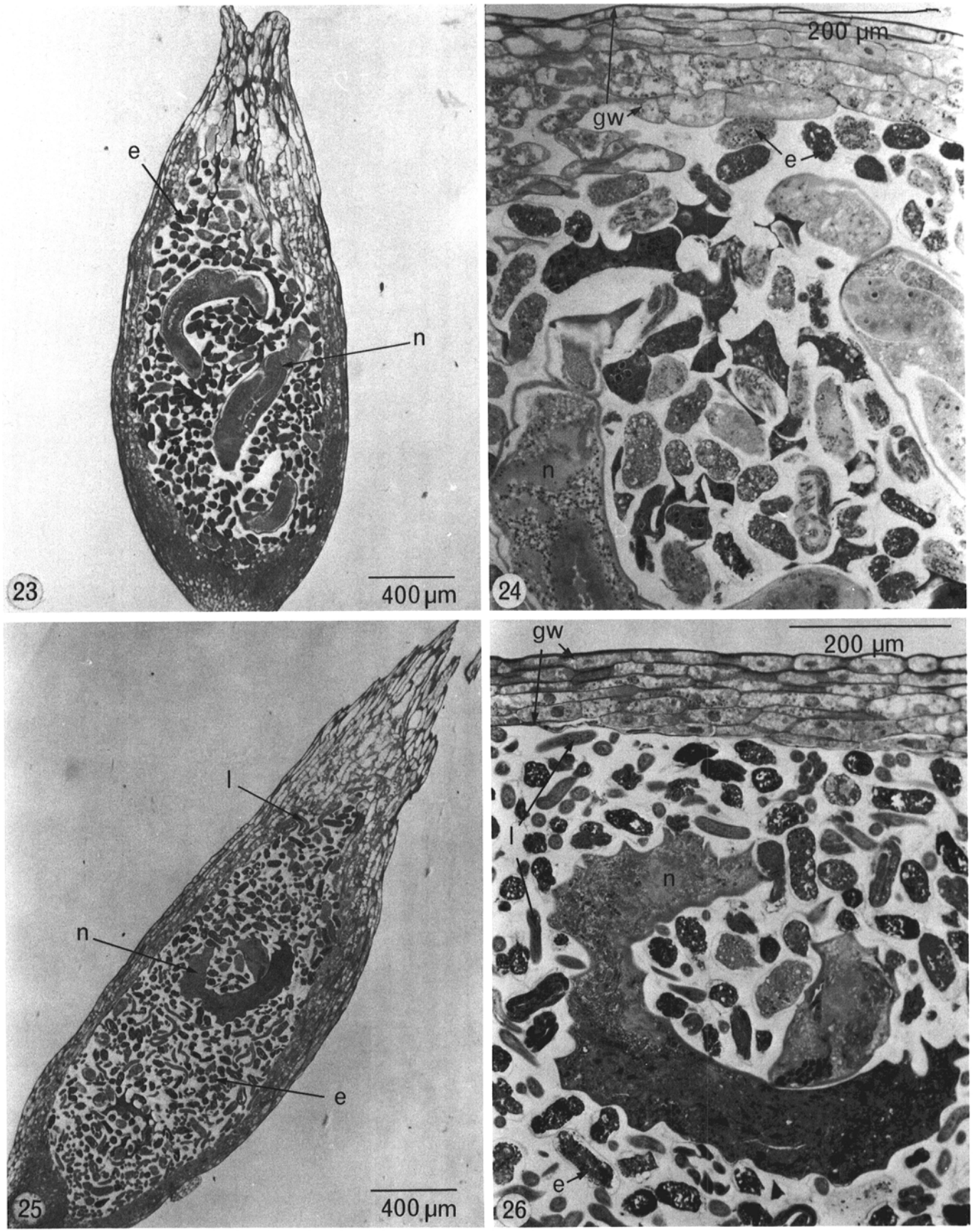
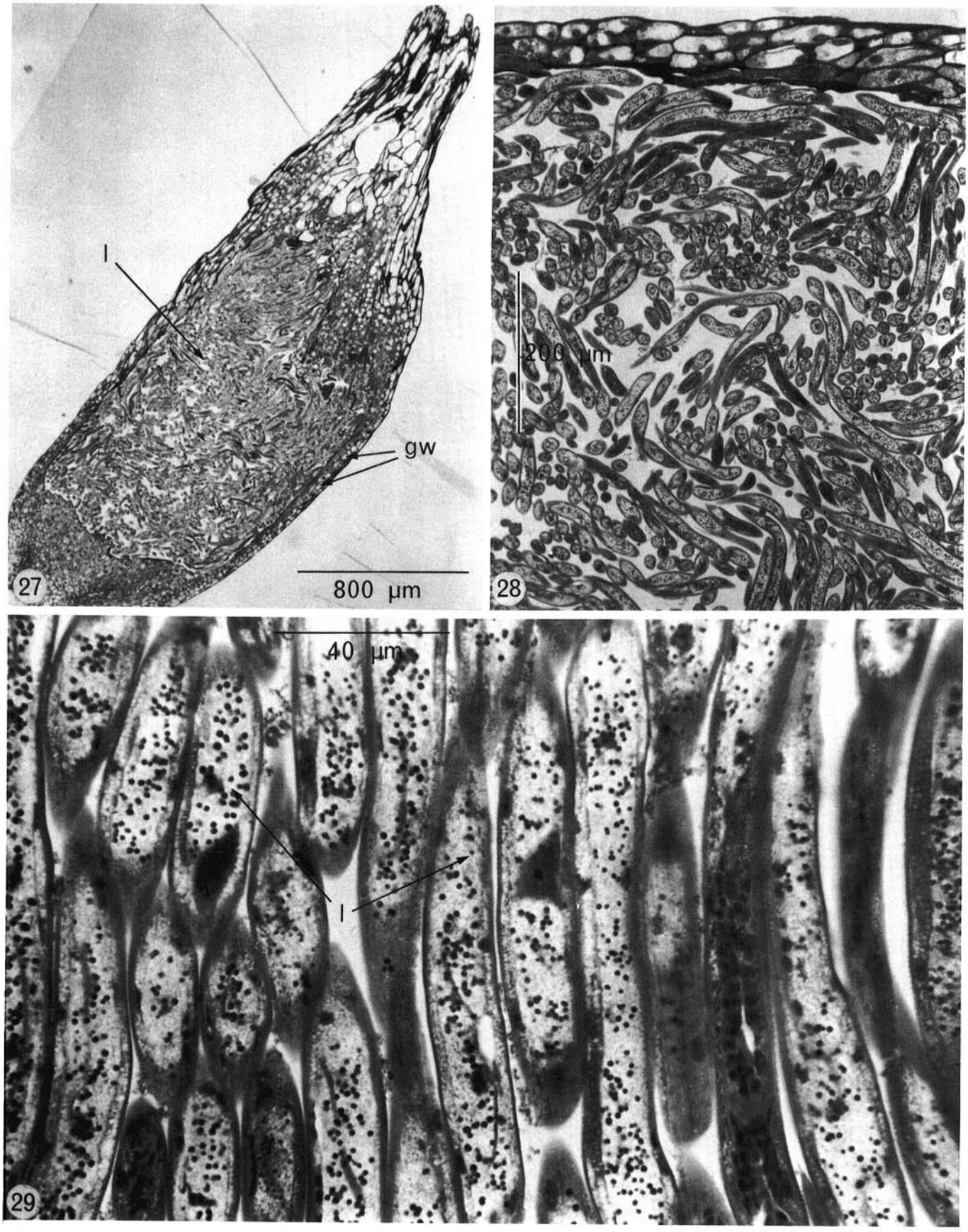


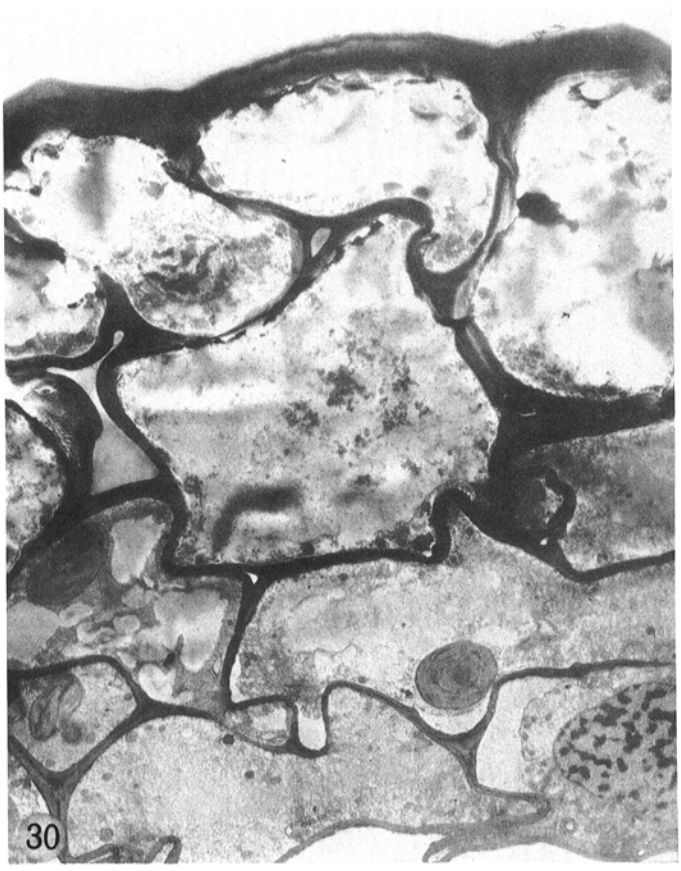

**Figs. 19–22.** Photomicrographs of sections through galls harvested in October. **19** and **20,** Younger gall, showing (Fig. 19) developing nematodes (n) in gall cavity before egg laying and, at higher magnification (Fig. 20), the nature of the cells in the gall wall (gw). **21** and **22,** Slightly older gall, showing nematodes in the gall cavity (Fig. 21), some of which have started to lay eggs (e), and, at higher magnification (Fig. 22), the nature of the cells in the gall wall.

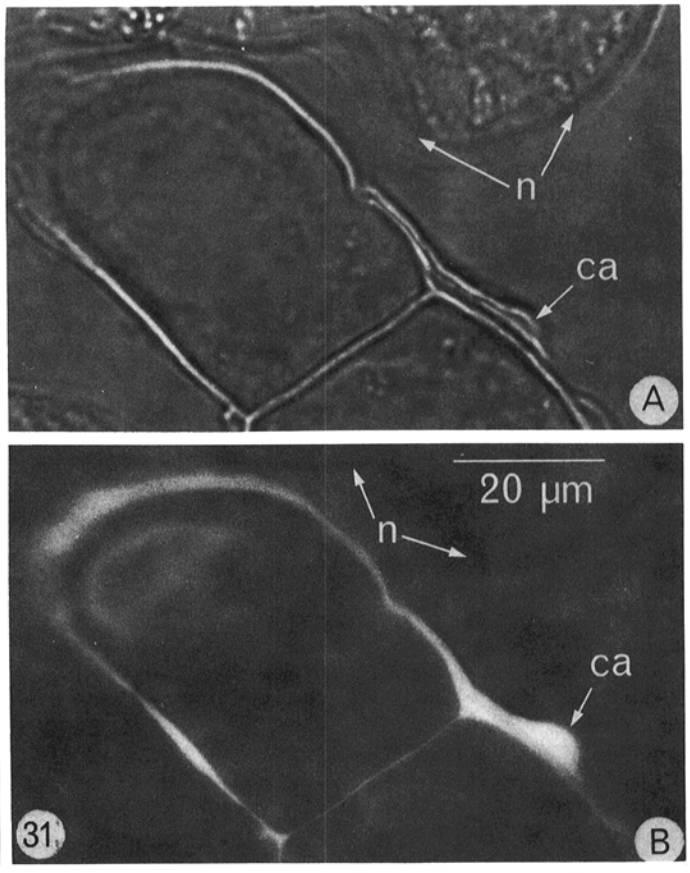



**Figs. 23–26.** Photomicrographs of sections through galls harvested in early November, showing: **23** and **24,** nematodes (n) that had laid large numbers of eggs (e) and, at higher magnification (Fig. 24), the nature of the cells in the gall wall (gw). **25** and **26,** Slightly older gall showing a moribund female nematode surrounded by eggs and freshly hatched nematode larvae (l) and, at higher magnification (Fig. 26), the nature of the cells in the gall wall.




**Figs. 27–29.** Photomicrographs of sections through galls harvested early in November and containing only infective dauer larvae (l) that will become anhydrobiotic as the seed gall dries out. **27,** Longitudinal section through the whole gall at low magnification, showing gall cavity full of larvae and thinness of the gall wall (gw). This is more apparent at higher magnification (Figs. 28 and 29). Larvae at this stage have not yet become coiled in their anhydrobiotic posture but tend to lie parallel in close proximity to each other (Fig. 29).




**Figs. 30** and **31. 30,** Electron micrograph of a section through a gall harvested early in November, showing the loss of cytoplasmic contents of many of the cells in the gall wall. **31,** Photomicrographs of cells lining the lumen in close proximity to feeding nematodes (n) after treatments with 0.05% aniline blue in 0.15 M $K_2HPO_4$ and showing the presence of callose (ca); **A,** under normal transmitted light; **B,** under ultraviolet light and exhibiting fluorescence due to use of a Zeiss filter combination specific for the 410-nm aniline blue excitation peak.

masses of fibrillar material, probably degenerating cytoplasm. With continued degeneration, the collapsed cells become compressed into a dense layer on the periphery of the cavity. Ultimately, complete dissolution of the remaining cytoplasm and cell walls results in enlargement of the cavity to accommodate the developing nematodes.

Throughout this period of gall development, the outermost cell layers remain intact, preserving the structure of the galls and thereby ensuring added protection for the larvae during summer. The directional growth of the galls is accompanied by elongation of the outermost cells, presumably keeping pace with the enlargement of the cells adjacent to the cavity. This elongation is particularly noticeable towards the apex of young galls but becomes more general as the galls age.

Despite the change in form and function of cells across the gall wall, we found no clear demarcation of tissues into a "rind" surrounding a rudimentary endosperm, as some workers have suggested (5,23). Rather, a gradual reduction in the influence of nematodes at increasing distance from the cavity appeared to occur. The production of callose is perhaps significant in view of the suggested role of these substances in sealing off damaged areas (7) after being produced in response to mechanical damage (8,9,11), plasmolysis (8), temperature stress (25), and viral infection (24). In this case, the production of callose may be to the advantage of the nematode because it ensures preservation of the gall structure while the nematode still has substantial influence over the host and can direct it to synthesize the considerable amount of metabolites necessary for growth, development, and reproduction.

The balanced host-parasite relationship and the enormous potential for seed production makes ryegrass an ideal host for *Anguina*. Sufficient sites are provided to carry over extremely high populations of nematodes, but the survival of the grass is never seriously threatened. Nevertheless, nematode populations can fluctuate considerably between seasons depending on the proportion of galls that become colonized by *C. rathayi*.

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