Histopathogenesis of the Galls Induced by *Nothanguina phyllobia* in *Solanum elaeagnifolium*

J. A. Skinner, C. C. Orr, and A. F. Robinson[1]

Abstract: The histopathogenesis of the foliar galls induced by *Nothanguina phyllobia* Thorne in *Solanum elaeagnifolium* Cav. was examined via serial sections prepared from plant shoots at 11 time intervals (0.5–30 days) following inoculation. Nematodes infected the blades and petioles of young leaves surrounding the shoot apex. Hypertrophy and hyperplasia of the palisade, pith, cortical, and vascular parenchyma resulted in the formation of confluent leaf, petiole, and stem galls up to 25 cm^3 in volume. Externally, leaf galls were irregular, light-green, convoluted spheroid bulges distending the abaxial surface. Mature galls contained a cavity lined with parenchymogenous nutritive tissue comprising intercellular spaces and actively dividing hypertrophied cells. These cells contained granular cytoplasm, hypertrophied nuclei, and brightly stained large nucleoli. Vascular tissues were not discernibly affected during the early stages of gall development. As gall development progressed, however, vascular elements were often displaced and disoriented. The histopathology of this nematode indicates that *N. phyllobia* is a highly specialized parasite and, for that reason, is suitable as a biological control agent. *Key Words:* histopathology, nutritive tissue, hypertrophy, hyperplasia, biological control.

Nothanguina phyllobia Thorne (the silver-leaf nightshade nematode) causes extensive galls on the leaves, stems, and flowers of *Solanum elaeagnifolium* Cav. (silver-leaf nightshade), an important perennial weed species of the southwestern United States (24). Orr et al. (20) proposed using *N. phyllobia* for biological control of *S. elaeagnifolium*. Subsequent studies on the host specificity, distribution, virulence (22), and behavior (23) of the nematode have demonstrated its suitability as a biological control.

The wide range of host responses to plant-parasitic nematodes was extensively reviewed by Dropkin (2) and Endo (3). Nematodes of several genera—*Ditylenchus* (27), *Anguina* (9,25), *Paranguina* (11), and *Nothanguina* (7,20,24)—cause galls on various aerial parts. Parenchyma hypertrophy and hyperplasia account for most of the gall caused by each of these genera. Galls induced by *Nothanguina cecidoplastes* (Goodey) Whitehead on the leaves of the

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grass *Bothriochloa pertusa* (L.) A. Camus have specialized cell types (7); that may be the case also with the galls induced by *N. phyllobia*. This paper describes the histopathogenesis of the galls induced by *N. phyllobia* on *S. elaeagnifolium*.

MATERIALS AND METHODS

Galled *S. elaeagnifolium* foliage collected from the Lubbock, Texas, area was air-dried to less than 10% moisture and stored at 10 C. Nematodes were extracted by mixing galled plant material with water and macerating in a blender for 1 min. So that more nematodes could escape, macerated tissue was soaked in water for 4 h; leaving the material in suspension longer paralyzed or killed the nematodes. Larvae that emerged from the tissue were concentrated in water and used immediately for inoculations.

Rhizomes of *S. elaeagnifolium* were cut into segments having three potentially active nodes (ca 6 cm). Five segments were planted horizontally 3 cm deep in each of 20 clay crocks containing 3 liters of a sterilized Olton loam soil (55% sand, 21% silt, 24% clay). These crocks were then watered to excess and placed in a greenhouse with adequate light and temperature; shoots emerged 4–10 days after planting. Plastic cylinders (2 cm high, 3 cm diam) were pressed 1 cm deep into the soil surrounding the stem bases of 70 young shoots (1–7 cm tall). The soil surface within each cylinder was then inoculated with 2 ml of an aqueous nematode suspension (ca 40,000 infective larvae). High relative humidity was maintained by enclosing each crock for 5 days in a moistened plastic bag. Nematode movement on foliar surfaces was verified by observing 10 randomly selected plants under a dissecting microscope several hours after inoculation, both *in situ* and after staining with acid fuchsin lactophenol.

Shoots were harvested 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, 15, and 30 days after inoculation; five replicates were included for each interval. Uninoculated shoots of similar size and age were harvested for anatomical comparison. Harvested plants were fixed in formalin-acetic acid-alcohol (FAA) (12), dehydrated in a tertiary butyl alcohol series, and embedded in Paraplast (Sherwood Medical Industries, Inc., St. Louis, Mo.). Shoots were sectioned transversely or longitudinally with a rotary microtome at 15 μm and stained with safranin and fast green according to Johansen (12). The use of picric acid as prescribed by Johansen was omitted. At 30 days, freshly harvested infected leaves from 10 additional plants were hand sectioned with a razor blade and examined microscopically.

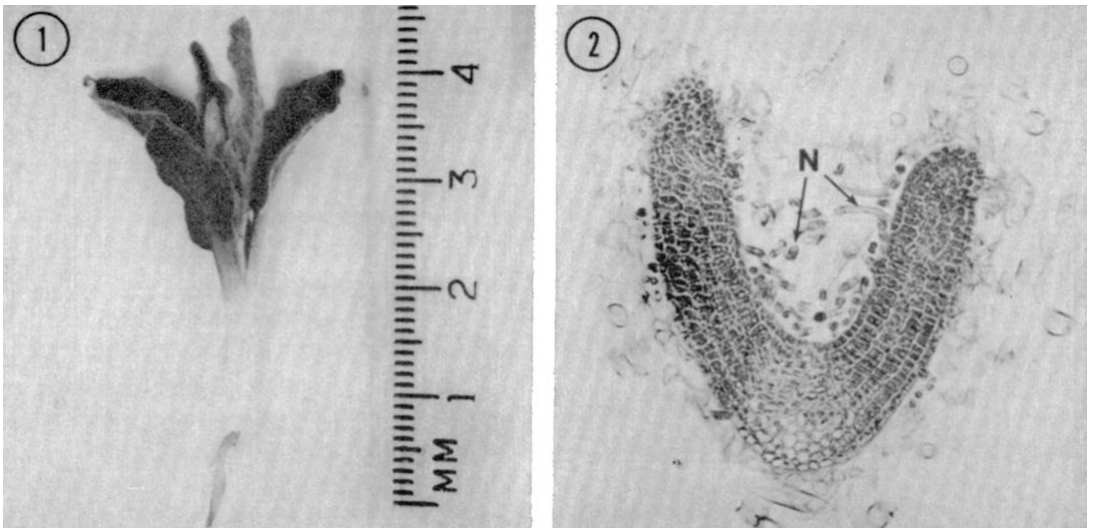
RESULTS

Normal anatomy: The shoot-apical system of *S. elaeagnifolium* was oriented spirally with a 3/8 phyllotaxy. In apical leaf blades, cells between the adaxial and abaxial epidermis were arranged into four distinct layers. A thick layer of palisade parenchyma (30 μm) occurred adaxially, and two thinner layers (each 20 μm thick) occurred abaxially. The remaining middle layer consisted of mesophyll and differentiating vascular tissue. During leaf expansion, the adaxial palisade remained distinct and the abaxial layers became disrupted by spaces. Stomata were always more numerous on the abaxial surface. The epidermis of leaves and petioles was single layered at all stages of development, whereas the epidermis of maturing stems was frequently double layered. All plant surfaces except those of the smallest apical leaves were covered by a dense pubescence of multicellular stellate trichomes.

0–1.5 days after inoculation: During this initial period, there were no discernible external pathological effects (Fig. 1).

At 0.5 days, ca 50 nematodes were observed moving among the trichomes on plant surfaces; larvae were becoming concentrated within apical leaf folds (Fig. 2). Rarely were nematodes observed within leaf tissue.

At 1 and 1.5 days, progressively more nematodes (up to 150) were found on plant surfaces. Most were within the apical leaf folds and about 5% were inside plant tissue. These nematodes penetrated cells of the epidermis and migrated through and between the cells below. Only cell walls in the immediate path of nematodes were destroyed. Necrotic tissue was rarely found, and no anatomical changes were observed within the surrounding tissue.



Figs. 1-2. *Solanum elaeagnifolium* 0.5 day after inoculation with *Northanguina phyllobia*. *1)* Shoot. ×1.4. *2)* Cross-section of an apical leaf showing nematodes (N) on adaxial surface. ×129.

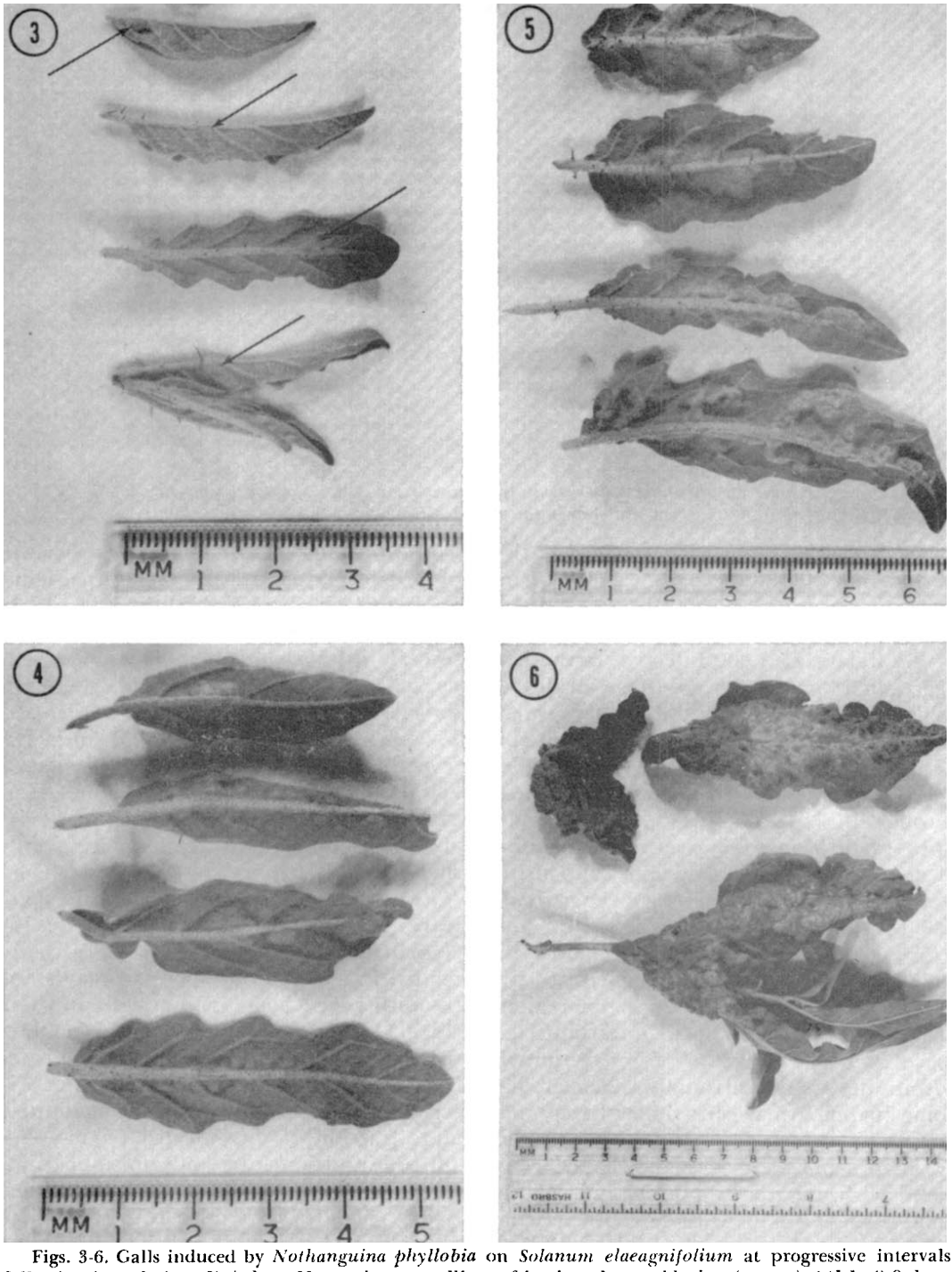
2–4 days after inoculation: During this period, minute swellings (< 2 mm diam) appeared along leaf veins, midribs, petioles, axillary buds, and stems (Fig. 3).

By 2 days, ca 1,000 infective larvae had aggregated among the 5–10 small leaves folded around the shoot apex. Larvae were frequently observed penetrating the epidermis of differentiating leaves and petioles (Fig. 7). Penetration usually occurred through the adaxial surface, and in most cases several nematodes used the same point of entry (Fig. 8). Nematodes within tissue were found in localized areas of the palisade and cortical parenchyma. The tunica and corpus of the shoot apex were seldom invaded, and nematodes rarely penetrated the middle layer of leaves or the stelar regions of petioles and stems. Small interconnected spaces (40–50 μm diam) formed tunnels within the tissue surrounding nematodes. Some parenchyma cells immediately adjacent to nematodes contained granular cytoplasm and were irregular-shaped compared with apparently normal cells of the same tissue. Cells with these characteristics are referred to herein as "granular cells."

After 3 days, expanding infected areas contained larger intercellular spaces (70–80 μm diam) and more nematodes. Some granular cells appeared hypertrophied and contained 2–4 densely stained nuclei in various stages of division; some nuclei contained 2–4 nucleoli (Fig. 9). Palisade cell plates were forming at various oblique orientations, resulting in a distortion and swelling of palisade layers. In leaf blades, periclinal epidermal duplication in the vicinity of nematode concentrations was observed in several instances. Some nematodes had molted, and developing ovaries were observed.

5–9 days after inoculation: Plants grew several centimeters taller during this period, and leaves continued to unfold and elongate. The minute swellings along leaf veins and on petioles and stems enlarged into small galls (> 5 mm diam) (Fig. 4).

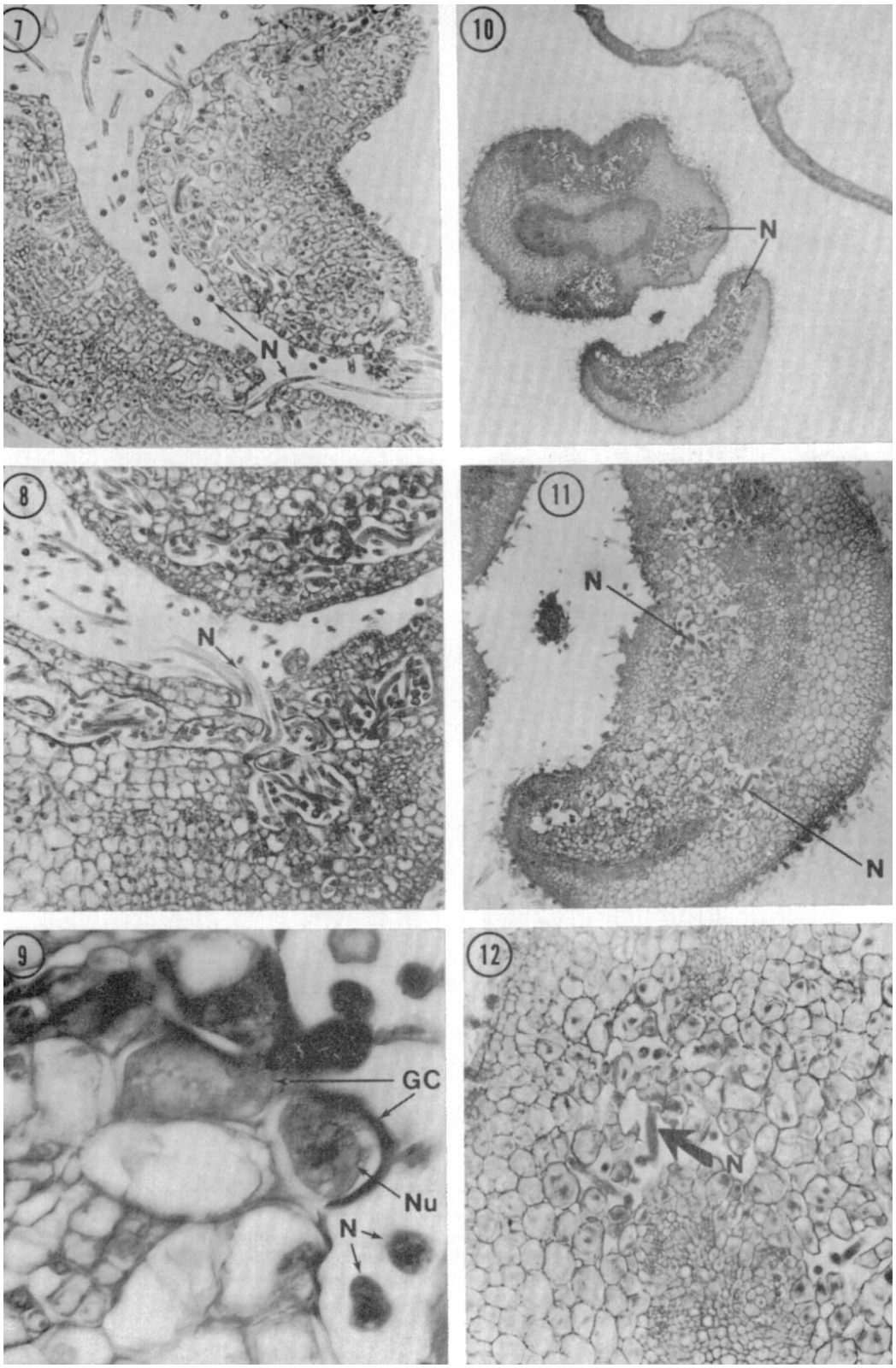
After 5 days, up to 30% of the tissues of leaves and stems were infected (Fig. 10). Nematode-infected areas occasionally extended into the middle layer of leaves and the pith parenchyma of petioles and stems (Fig. 11, 12). Parenchyma cells in these regions had granular cell characteristics, but most vascular elements were not discernibly affected. Granular cells made up a large fraction of the infected tissues (Fig. 13), and multinucleate and multinucleolate cells were more frequent than in normal tissue (Fig. 14). Granular cells were characterized by hypertrophy of the nucleus and the nucleolus, which had volumes respectively 10× and 25× those of apparently unaffected cells in the same tissue (Fig. 13). Staining anomalies were also noted: the cytoplasm, nuclei, and the nucleoli of granular cells respectively stained blue, dark purple, and bright red, while the same cellular components in unaffected cells stained



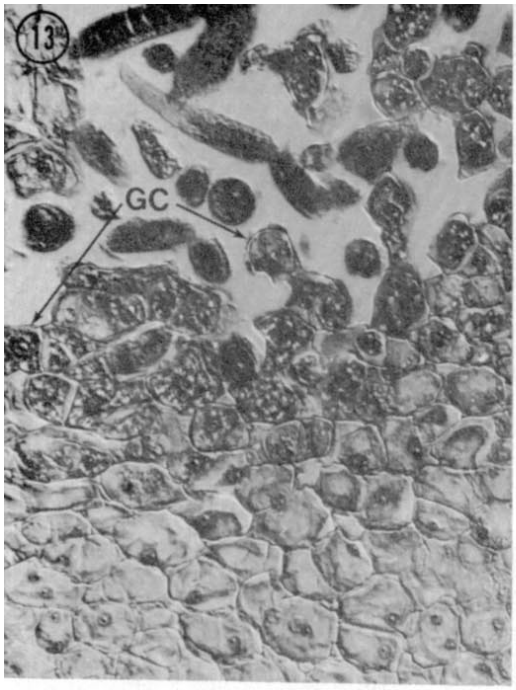
Figs. 3-6. Galls induced by *Nothanguina phyllobia* on *Solanum elaeagnifolium* at progressive intervals following inoculation. *3)* 4 days. Note minute swellings of lamina along midveins (arrows). ×1.1. *4)* 9 days. ×1.1. *5)* 15 days. ×0.9 *6)* 30 days. Note confluence of galling on leaves, petioles, and stems. ×0.4.

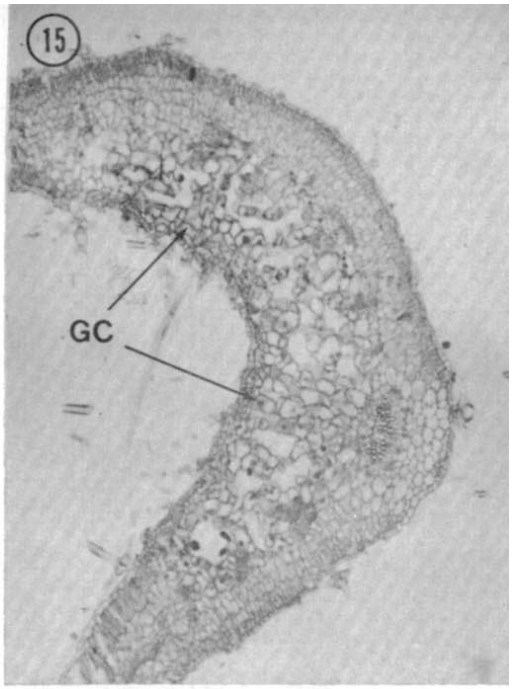
green, light purple, and dull red. Gravid females and small numbers of eggs were observed.

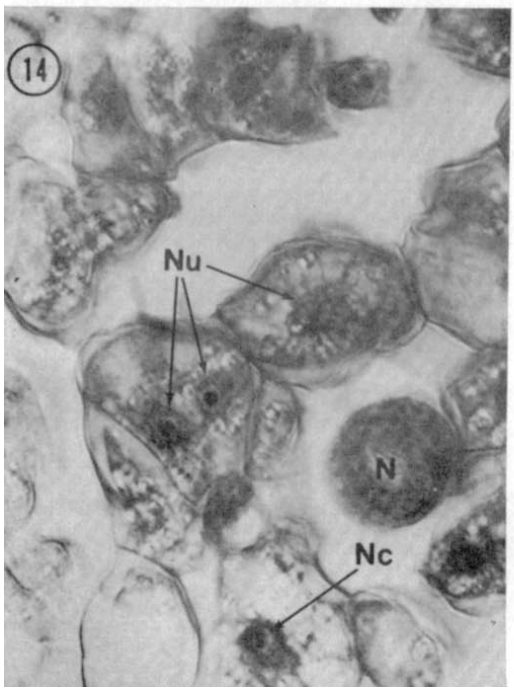
At 7 and 9 days, the central region of each gall consisted of a loosely packed tissue composed of intercellular spaces and actively dividing granular cells (Fig. 15). Within this tissue, 2,000 nematodes were observed; these included all developmental stages and many eggs. On the peripheries of

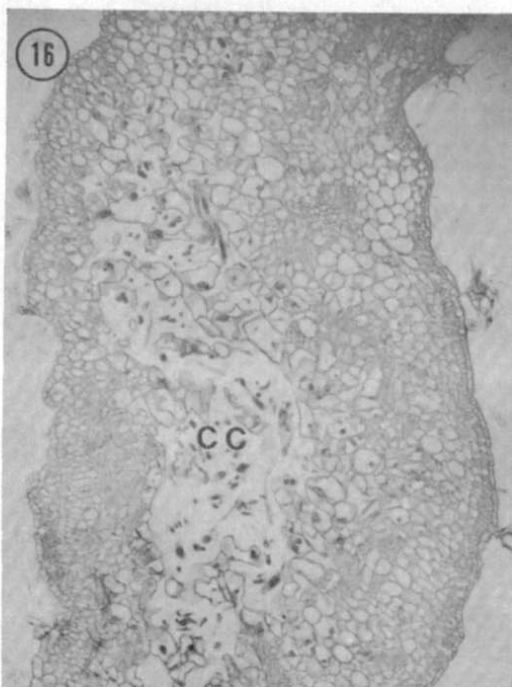


Figs. 7-12. Cross-sections of *Solanum elaeagnifolium* shoot regions infested by *Nothanguina phyllobia*. *7-8*) Penetration of epidermis by nematodes (N) 2 days after inoculation. ×115. *9*) Granular cells (GC) in lamina parenchyma 3 days after inoculation. Note nuclei (Nu) of quadrinucleate cell. ×730. *10-12)* Nematodes within tissue 5 days after inoculation. Note invasions of vascular region of petiole. Respectively ×15, ×46, and ×115.









Figs. 13-16. Cross-sections of developing *Solanum elaeagnifolium* galls. *13)* Granular cells (GC) 5 days after inoculation with *Nothanguina phyllobia* (Nomarski). Note granular appearance of cytoplasm. ×286. *14)* Granular cells with more than one nucleus (Nu) near nematode (N). Note hypertrophied nuclei and nucleoli (Nc). ×730. *15)* Granular cell tissue 7 days after inoculation. ×53. *16)* Foliar gall 15 days after inoculation. Note central cavity (CC) ×41.

galls, larvae were migrating into previously uninfected areas.

15 days after inoculation: Galled regions on leaves, petioles, and stems had assumed the appearance of convoluted spheroid thickenings up to 5 cm^3 in volume (Fig. 5).

In transverse section, galls measured 20–40 cell diameters in thickness (Fig. 16). Epidermal cells formed a continuous layer, and some were elongated periclinally up to 3× their normal size. Stomata and trichomes appeared normal. Below the epidermis was observed a discontinuous layer of palisade parenchyma and a region of normal-appearing spongy mesophyll 3–5 cell diameters thick. Centripetally, large granular cells formed a loosely packed network that lined and extended into an irregularly shaped central cavity. Nematodes of all stages were observed inside the cavity and among the cells surrounding it. Vascular tissue was disoriented and was located adaxially and abaxially to the cavity (Fig. 17).

30 days after inoculation: Galls varied in volume from 3 to 25 cm^3. Leaf galls had become irregular spheroid bulges (0.5–2 cm in diam) that distended the abaxial surface, particularly along midribs and secondary veins. Galled petioles and stems were similarly swollen. Often, the confluence of large galls on adnate organs made identification of original plant parts difficult (Fig. 6).

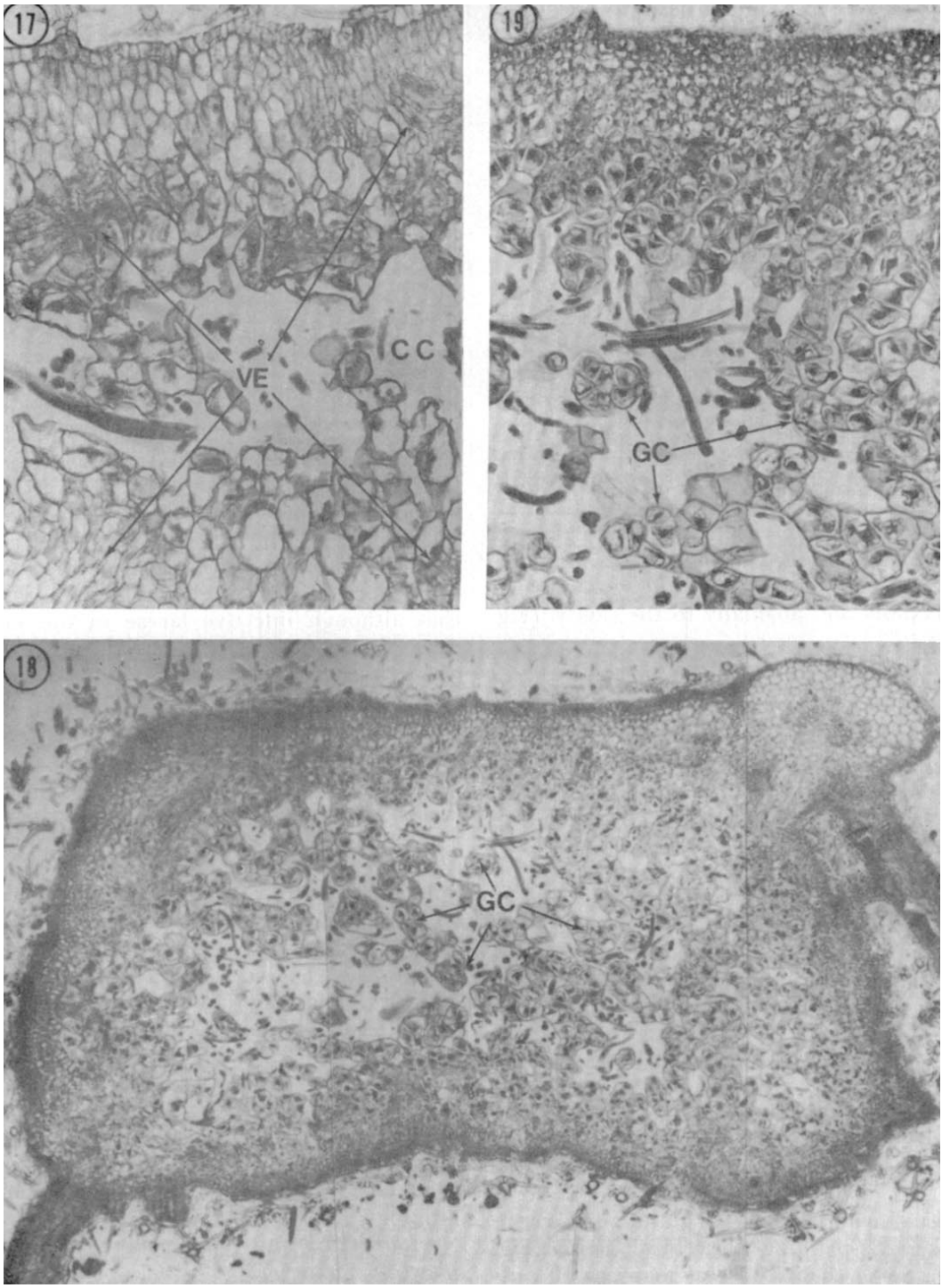
Thirty-day galls were anatomically similar to 15-day galls. However, the central cavity had enlarged (up to 1 cm in diam) and the wall of tissue enclosing the cavity had thickened (up to 1 mm in diam) (Fig. 18). The outer 200 μm of subepidermal tissue was dark green in fresh tissue and contained numerous chloroplasts. Interior spongy mesophyll and granular cell tissues, in contrast, were yellow and contained few or no chloroplasts. Numerous interconnected hyphoid chains of granular cells were observed spanning the central cavity (Fig. 19). Some chains were as many as 10 cells long. Nematodes were concentrated in granular cell tissue and few were found within the chloroplast-containing peripheral zone. Second-generation females had matured, active egg deposition was occurring, and nematode populations in many galls exceeded several hundred thousand.

DISCUSSION

The ascension of stems by foliar and floral-feeding nematodes and their aggregation within apical leaves and inflorescences have been documented for numerous nematode species (1,14,24). Infective larvae of *N. phyllobia* also ascended stems and continued to accumulate in the apical region as long as high relative humidity maintained a moisture film on foliar surfaces. Tissue invasion by *N. phyllobia,* however, was slow compared with that reported for some foliar-feeding nematodes. Several species penetrate host tissue less than 7 h after inoculation (10,17,25,26). *Nothanguina phyllobia* larvae were not found within tissue until ca 24 h after inoculation, in spite of up to 1,000 larvae located on apical surfaces. Numbers found inside tissue were not appreciable until 48 h. This information correlates well with a phenomenon we have repeatedly observed in the field, viz., that although infective larvae in the soil revive from anhydrobiosis in a short period ($<$ 8 h) and quickly ascend *S. elaeagnifolium* stems (at rates $>$ 6 cm/h), continuous moisture is required for at least 72 h before galling is initiated (22).

Cytochemical, mechanical, or behavioral factors may explain the delay of host penetration by *N. phyllobia. Aphelenchoides* spp. have been reported to enter plant tissue only through stomata (15,26), but stomatal entry by *N. phyllobia* was not observed. The leaves and petioles in which massive nematode galls were initiated were frequently $<$ 5 mm long when inoculated, and only limited differentiation had occurred. In the greenhouse and in the field, expanded leaves were not invaded, suggesting that galls are initiated only in actively growing undifferentiated tissues. Similar observations were reported for other foliar-feeding nematodes: *Ditylenchus dipsaci* (Kühn) Filipjev (27), *Anguina agropyronifloris* Norton (19), and *Anguina microlaenae* (Fawcett) Steiner (5).

Infective larvae molted into adults that produced eggs within 7 days. In less than 1 month the eggs gave rise to several hundred thousand progeny. This high biotic potential indicates a substantial nutrient supply. The granular cytoplasm and pronounced nuclear activity observed within



Figs. 17-19. Cross-sections of maturing *Solanum elaeagnifolium* galls. *17)* 15 days after inoculation with *Nothanguina phyllobia*. Note that vascular elements (VE) are located on adaxial and abaxial sides of central cavity (CC). ×115. *18-19)* Cross-section 30 days after inoculation. Note granular cells (GC) lining the wall of tissue surrounding and extending into the central cavity. Respectively ×48 and ×110.

granular cell tissue (Figs. 9, 13, 14, 18, 19) are characteristics that were observed in light-microscope investigations of the nutritive tissue in the galls of numerous cecidozoans (2,6,7,8,16,18,21,27,28). Closer examination of several nematode and arthropod species with the electron microscope revealed that the granular appearance of cytoplasm resulted from mitochondrial and ribosomal proliferation, cell wall ingrowths, and a dense cytoplasmic matrix similar to that found in transfer cells (4,13,21). Enlarged nuclei and nucleoli, similarly, are associated with the active synthesis that occurs in transfer cells (4). The exceptional biomass of *S. elaeagnifolium* galls (Figs. 6, 18) supports the premise that *S. elaeagnifolium* granular cells are transfer cell-like in nature and make up a tissue that functions as a physiological sink. The mechanism by which *N. phyllobia* triggers the development of granular cells is not known.

Nothanguina phyllobia is a highly specialized parasite. It is host specific and has a high biotic potential. In addition, it has a histospecific and exceptionally pronounced effect on the morphology of its host. In the early stages of gall development, nematodes migrated paradermally, seldom invading vascular regions. By the time nematodes invaded vascular regions granular cells derived from undifferentiated parenchyma had developed within peripheral tissues. Although disoriented and displaced by proliferated ground tissue, vascular elements were functional. Even in severely infected plants, the shoot apex was seldom destroyed.

In terms of plant-nematode associations, *S. elaeagnifolium* is a highly susceptible host for *N. phyllobia*. Cellular responses by the plant result in rapid nematode development and reproduction; necrosis is rare. Larvae migrate into uninfested portions of the lamina throughout gall development. The nutritive zone is massive compared with that of most gall-forming nematodes, and although chlorophyll occurs in the outer layer of the wall surrounding the central cavity, it probably contributes but a small fraction of the photosynthates required for gall development. If that is correct, the galls induced by *N. phyllobia* on *S. elaeagnifolium* are constructed largely from translocated assimilates and impose a considerable energy drain on the plant.

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