Host-Parasite Relations of Nacobbus Batatiformis and the Sugar Beet and Other Hosts[1]

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Introduction

Detailed investigations of the parasitic behavior and host reaction have been made of *Nacobbus batatiformis,* Thorne and Schuster in roots of sugar beets and other plants to elucidate the biology of this nematode. This pathogen causes an economically important disease in western Nebraska and elsewhere. Some studies have been made and reported in 1956 (7,9)[3] but additional information has been obtained on histopathology and cytopathology to more fully understand the nematode's activities. This paper embodies these studies.

Because the genera *Meloidogyne* and *Heterodera* have received more attention than other genera and because certain similarities exist, it seems desirable to compare and contrast these genera with *N. batatiformis.* Studies on the cytopathology and histopathology of *Meloidogyne* and *Heterodera* in plant roots are well reviewed in the literature (1,4) and a detailed review will not be presented here.

Materials and Methods

N. batatiformis used in these experiments was taken from a population collected in Scotts Bluff County, Nebraska. These cultures had been maintained on *Beta vulgaris* L. grown in benches, flats, pots, or petri dishes.

Other nematode species compared with *N. batatiformis* included *Meloidogyne hapla* Chitwood, and *Meloidogyne incognita* Chitwood obtained from infected sugar beets grown in Scotts Bluff County, Nebraska and maintained for several years in the greenhouse on this host. *Heterodera schachtii* Schmidt was obtained from the same area and maintained on greenhouse grown sugar beets.

The effects of the nematodes on plant growth were determined in greenhouse and tissue cultures. In greenhouse tests, sugar beet and other seeds treated with 10% Purex (a commercial bleach containing 5.25% NaOCl) for 20 minutes were

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[3] Numbers in parentheses refer to literature cited.

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sown in wooden flats of soil that had been steam sterilized at 250° F for 2 hours. Infections were obtained by adding to the sterilized soil ample amounts of inoculum consisting of chopped roots from the greenhouse subcultures. Seeds of sugar beets and other crops were sown at the time of inoculation.

Tissue cultures for the nematode study were obtained from root tips of sugar beets and other crops. The apices of excised roots or intact seedlings were placed aseptically in petri dishes containing White's medium as modified by Skoog and Tsui (8). Each dish contained one excised root or intact seedling and about 35 ml of the 1% agar medium. The seeds were treated 15 minutes with 10% Purex, rinsed in sterilized distilled water, and transferred to moist filter paper to germinate. When the seedling root was about 3 cm long, 1 cm of the apex was excised and transferred to the artificial medium. Egg sacs of the nematodes were sterilized by agitating 4 minutes in a test tube containing 5 ml of 10% Purex. This procedure broke up the egg sacs and surface sterilized the eggs (6). When cysts were used, the cyst was first broken and the released eggs were sterilized by the method just described. The suspension of eggs was transferred into a separatory funnel containing 100 ml of sterilized distilled water. The nematode eggs that settled in about 25 minutes were drawn off (with minimum liquid) through the base of the funnel. These eggs were then transferred to the tissue culture agar medium upon which were placed excised root tips or intact seedlings of sugar beets or other plant species. Temperatures ranged from 70-75° F.

Standard histochemical methods were followed in processing the infected roots (3). The samples for histological studies were collected at periodic intervals. Then the roots were fixed in FAA (90 ml 70% ethanol, 8 ml glacial acetic acid and 4 ml formaldehyde) or FPA (90 ml 50% ethanol, 8 ml propionic acid, and 4 ml formaldehyde). Dehydration and paraffin embedding were accomplished in an Autotechnicon with tert-butanol as solvent. Sections were cut 10-15 μ and stained with appropriate reagents. The stains used with paraffin sections were Harris' haemotoxylin, safranin and fast green, Johansen's quadruple stain or Feulgen's reagent. Johansen's quadruple stain proved very useful in illustrating changes in the cell walls, nuclei, and cytoplasmic contents.

For whole mounts, roots were boiled in lactophenol-acid fuchsin, rinsed in running tap water to remove excess stain, then cleared in lactophenol. Root portions containing the parasite and considered suitable for histopathological study were re-

moved for further processing and paraffin embedding. In other instances, unstained roots with easily recognizable symptoms (galling, necrosis) were embedded in paraffin preparatory to sectioning and staining. Observations and photographs of prepared materials were made with light, phase, or ultraviolet microscopes. Tissue cultures were observed through a phase microscope equipped with suitable objectives. Observations of egg hatching and larval development were made in BPI watch glasses using the low or high power objectives of the phase microscope.

Experimental Results

In studying the life cycle of *N. batatiformis*, examination of hundreds of eggs did not reveal any molting of the larvae prior to hatching. These eggs hatch soon after embryonation: the larvae were seen puncturing the egg cuticle and eclosion resulted through a slit in the egg cuticle. The larvae make a circle or figure 8 during movement in the egg before eclosion. The larvae hatching from the eggs measure about 380 μ in length and are the infective stage. This stage is considered the first larval stage. In BPI dishes, this stage was seen to molt and assume a length of about 500 μ. However, the larval stage does not take on the brownish coloration found in the root tissue after penetration and feeding. This stage in the root is the "C" stage which perhaps is the second larval stage. This stage then assumes a spiral stage about 800 μ in length and is considered the third larval stage; it has 1-2½ coils. Presumably the fourth and adult stages are sedentary, while the three early stages are motile.

Sexual dimorphism is pronounced in *N. batatiformis*. The male retains its eel-shape. In tissue culture, an advanced larval stage was observed molting while attached to the surface of an excised sugar beet root. Upon molting, the larva proved to be male. Molting was similar to *Pratylenchus* rather than to *Meloidogyne*. In the latter, the male is coiled in a sausage shape cuticle. The male apparently uses its stylet and pressure of its body to release itself from the fourth larval skin. *Nacobbus* is closely related to *Pratylenchus* rather than *Meloidogyne* so the mode of molting of the male was not unexpected.

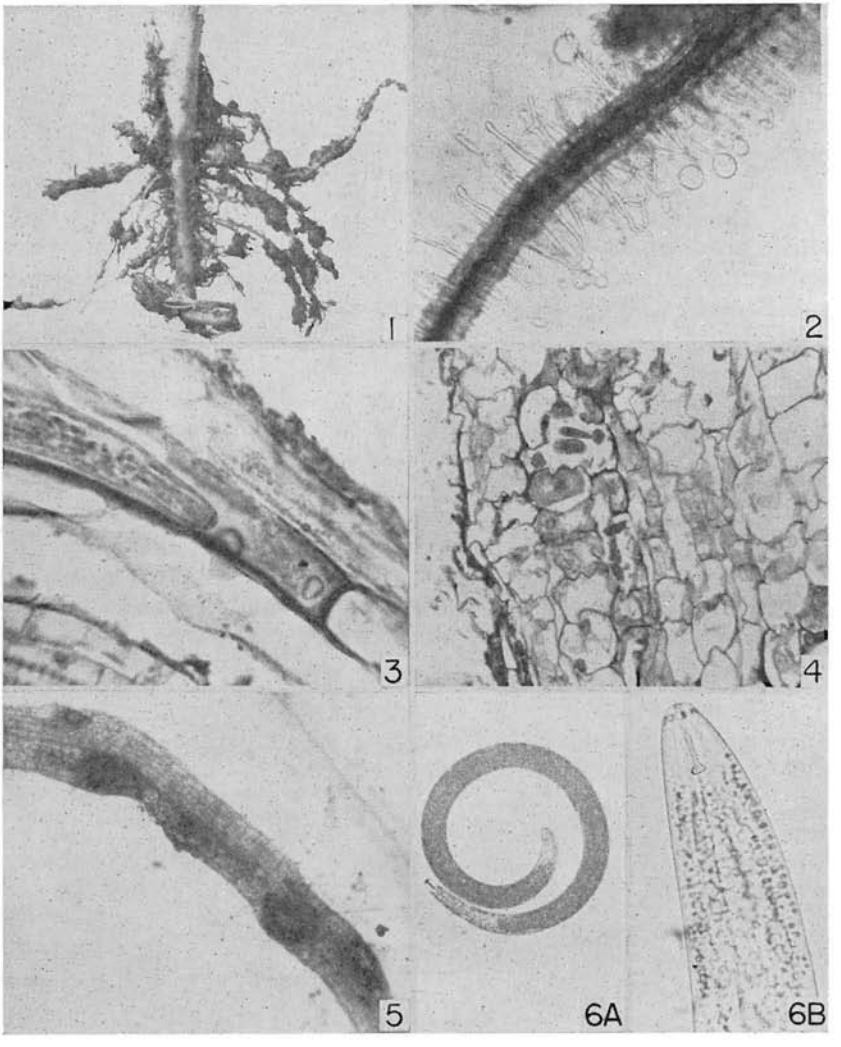
In tissue cultures and in field and greenhouse grown sugar beet roots, (Figure 1) *N. batatiformis* is found most commonly in the cortex as contrasted to the stele for *Meloidogyne* and *Heterodera*. Hypertrophy of the epidermal and cortical cells occurred within a few days after surface feeding or penetration.

In tissue culture, the nematode larvae caused such pronounced hypertrophy and associated loosening of cells of the surface and cortical cells as to induce callus-like effects. In soil-grown roots such effect is not evident because the tissue remains more compact. The epidermal cells tend to become spherical in infected tissue culture roots; this causes a loosening of adjacent cells. The nematode larvae frequently feed on root hairs and cause hypertrophy of the tip, base or center part of the root hairs (Figure 2). Root hairs in non-galled areas show these effects but are usually inhibited in the galled areas.

The nematode was not restricted to the root tip as larvae were found up and down the root a few days after inoculation. Frequently the root tip would become swollen and further apical growth inhibited due to entry and penetration through the root tip. Apparently meristematic activity of the root tip was inhibited, but some lateral swelling of the root tip resulted. The resulting gall, pendulum-like, at the end of the root occurs more frequently in the tissue culture than in soil-grown roots. Often larvae were located near the base of the lateral root where entry occurred probably through the separation of cortex and epidermis resulting from emergence of the branch root.

The larvae tend to penetrate the root intracellularly. This is noticeable from prepared sections and from destruction of cells as evidenced by necrosis along the path of the larvae. This latter effect is quite evident in whole mounts of roots boiled in lactophenol acid fuchsin. Microscopically the injured areas appear red to reddish brown. In this stain, the epidermal cell walls in the necrotic spots appear brown as do the nuclei. The cell walls in the gall stain a dark brown to purplish brown; the walls fluoresce white under ultraviolet light. The larvae, while migrating through the root, had apparently broken down end walls and passed through rows of cells (Figure 3). The destruction of cells is more severe than that of *Meloidogyne* larvae which migrate largely intercellularly. Intracellular migration can be adequately demonstrated, for at times the larvae are coiled within a single cell which reacts to its invader by thickened necrotic walls. These walls fluoresce with ultraviolet light. The larvae can be seen passing through the cells with the nematode embedded in the cell causing a rupture due to the nematode's movement or increase in size.

Initially the larva orients itself in the cortex parallel to the long root axis; its anterior may be directed toward or away from the root tip. After the first larval stage, the coiled or "C" shaped stages do not orient themselves to the root axis. Associated with



Figures 1-6.—1.) Sugar beet root galls induced by *N. batatiformis.* 1 X. 2.) Effect of *N. batatiformis* feeding on root hairs of sugar beet root. 155 X. 3.) Intracellular penetration of root by larvae; note nucleus near nematode's anterior. 1820 X. 4.) Cavities in small gall showing hypertrophy and increased granularity of cells. 366 X. 5.) Young root galls showing necrotic areas induced by the nematods. 105 X. 6A.) Advanced coiled larva (300 X) in necrotic spots illustrated in Figure 5; note the heavy concentration of fat globules in this and Figure 6B which shows anterior portion of the coiled larva. 4000 X.

this "C" or coiled stages are the tunnels of broken cells in the cortex of the young roots (Figure 4). These cavities were evident by the presence of necrotic spots in small galls (Figure 5). The necrotic spots readily took up acid fuchsin stain. Teasing of the necrotic spots in young galls yielded usually more than one "C" or coiled dark brown larva (Figure 6A & B). Upon sectioning of these small galls, larvae could be found in the cavities. Breakdown of several cells results in formation of the cavity which appears to be devoid of cellular material. There seems to be little evidence of chemical dissolution of cell walls so pronounced in older infections. The cavity is formed by mechanical tearing, pressure, and feeding on the cortical cells (Figure 7). The faces of the cell walls are indented as though pressure were exerted centrifugally. The cavity containing the nematode seems to enlarge as the nematode grows. Granular residues, precipitates from the cytoplasm, or excreta from the nematode are deposited in necrotic cells or on the outer walls of the cavity. The "C" and coiled larvae are gorged with globules (fat as determined by Sudan III test) so that an oblique angle between the anterior position and the intestine, so characteristic of the hatched larval stage, is masked due to presence of globules and the brownish cast of the advanced larvae.

The immediate reaction of root tissues to infection is necrosis and hypertrophy. The first macroscopic change is the necrosis and hypertrophy of the cortical and epidermal cells. In small galls, 7-10 days after infection, this effect is noticeable. Sectioning of these galls shows that galling is due primarily to hypertrophy of the cortical cells. The number of rows of cortical cells was similar in galled and non-galled roots. Hypertrophy is not confined to cells adjacent to larvae, but extends also to those some distance away. Cells of the endodermis may show necrosis and some hypertrophy but this effect is usually not in the central cylinder.

Usually the female tends to lie parallel to the long axis of the root and does not affect the vascular tissue as viewed through bright light. When the female lies perpendicular or obliquely to the longitudinal axis, it affects the vascular tissue; with bright light this appears more physical than chemical. Under such circumstances, the endodermal cell walls fluoresce white as though affected. The phloem and xylem elements, although separated from the nematode by only a layer or two of cells, seem singularly free from necrosis, hypertrophy, and rupture. However, fluorescent studies show that the alteration of cellulose that glows white

and has been seen to be characteristically associated with nematode-induced necrosis and cell wall changes, is found surrounding the nematode; fluorescence is apparent in the phloem and in the xylem too, but difficult to assess in the xylem which fluoresces strongly naturally. The cells lining the cavity in the small galls increase in their volume of cytoplasm and granular content. Large numbers of larvae enter a root in the same area, but apparently they are dispersed because clusters of adult females are usually not observed. In contrast, several *Meloidogyne* larvae sometimes occupy a single gall and cause a proportional increase in swelling up to a point. It has been assumed that the advanced larval stages of *N. batatiformis* leave the necrotic roots and infect other roots (9). The wounds and necrosis interfere with the function of the smaller branch roots or the tap roots. In the field, complete loss of stand due to *N. batatiformis* occurs. Plants that remain never attain good growth, but are stunted.

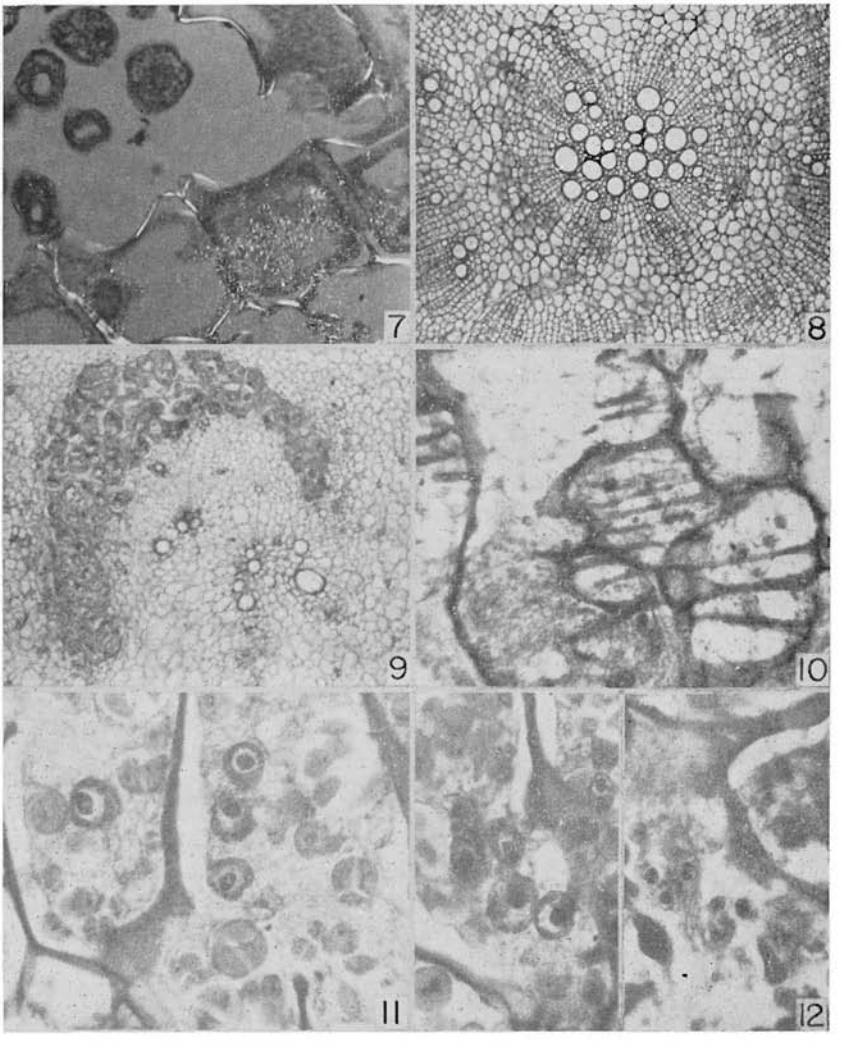
Establishment of the nematode in a permanent site with its entire body within the cortex initiates a series of phenomena. One effect is the production of lateral roots in the galled areas. This is of interest because the lateral roots arise from the pericycle and the nematode is localized in the cortex. The presence of the nematode seems to stimulate mitotic activity in the pericycle which is some distance away. Stimulation of lateral roots on the galls is a characteristic morphological symptom of *N. batatiformis*. The numbers per gall range from several to over 50. These lateral roots arise from all parts of the gall. In contrast, lateral roots arise in two rows from the normal, diarch sugar beet tap root (Figure 8). The presence of a nematode in a nearby cell can cause the lateral root to alter its original path or even to continue down the cortex parallel to the vascular area.

In tissue culture, *N. batatiformis* affects the root in a way somewhat analagous to the proliferation of lateral roots. This effect is the frequent induction of stem buds (rootlings). The buds arise from the pericycle in a manner similar to lateral roots. The vascular tissue leading to the bud base is similar in morphology to that in the lateral root. The leaves on these buds have typical leaf appendages. The induction of stem buds may be due to an increase in the adenine/indoleacetic acid ratio which is known to differentiate callus into leaves and stems (8). An attempt is being made to grow these buds to mature plants.

Another characteristic histological symptom induced by *N. batatiformis* is the syncytium which is comparable to the giant cell complex of *Meloidogyne*. The syncytium induced by the feeding of *N. batatiformis* becomes a highly granular, deeply staining, multinucleate, protoplasmic mass of what earlier had been hundred of cells. It is formed by the merging of protoplasts due to the gradual dissolution of cell walls. Despite the progressive incorporation of neighboring cells into the syncytium, many cells still tend to retain their individuality. The changes begin in a localized area and extended into surrounding tissues. The syncytium is poorly delimited, merging gradually with the normal tissue. The syncytium typically is located entirely within the cortex and is bounded by the endodermis, or on occasion by the xylem elements. In cases when the nematode is located very near the stele, the syncytium is crescent-shaped in transection with the concave side toward the stele (Figure 9). The general shape of the syncytium is oval or spindle with its long axis parallel to the main axis of the root. The anterior portion of the nematode is embedded in one end of the spindle about one-fourth the total length of the syncytium. The greatest diameter of the syncytium is not necessarily closest to the nematode's stylet. Cell wall dissolution occurs most rapidly and in greatest intensity adjacent to the nematode's head. The damage to the cortex may extend a few mm from the feeding point. The nematode, when in its permanent position with its head in the syncytium, does not need to move for feeding. Anteriorly from its stylet there appears to be a channel into the syncytium. It may draw its nutrition via this route (Figure 19).

The syncytia, which result from hyperplasia and hypertrophy, vary in size and content depending upon age and stage of development. They have been observed to reach up to 3 mm in length and about 2 mm in diameter six to eight weeks after inoculation. The walls of the syncytial cells thicken. Upon dissolution certain portions dissolve differentially giving the appearance of scalariform type xylem tissue (Figure 10). Dissolution of cell walls first causes perforations which enlarge until the entire walls are dissolved.

In the earliest stages of syncytium development, the larva causes characteristic symptoms which are retained later. The initial cells become hypertrophied. In these cells, the nucleus becomes hypertrophied and the nucleolus becomes enlarged and measures 5 times larger than in normal adjacent cells. The nucleolus stains a deep red in Johansen's quadruple stain and

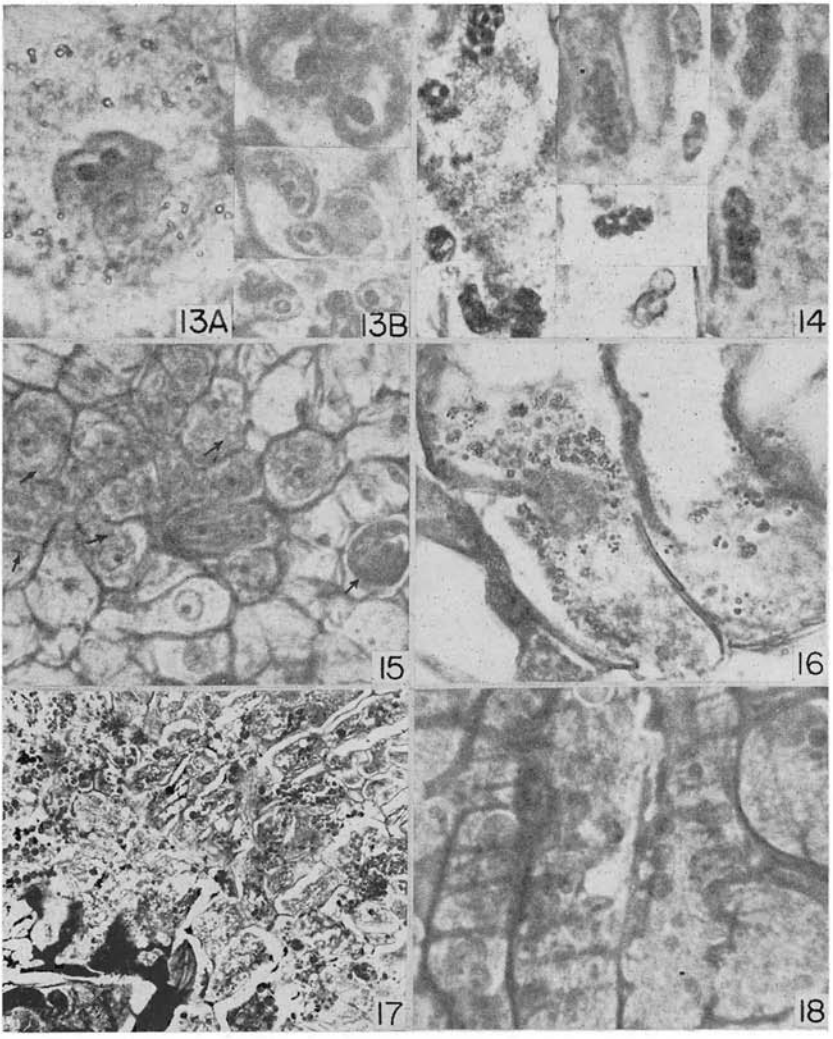


Figures 7-12.—7.) Cavity showing cross sections of nematodes and evidence of mechanical pressure on faces of cells lining the cavity. Polarized starch grains are shown in the three lower cells. 700 X. 8.) Transverse section of a normal diarch sugar beet root. 400 X. 9.) Transverse section of *Nacobbus*-infected sugar beet root showing crescent-shaped syncytium with the concave side toward the stele. 420 X. 10.) Differential dissolution of syncytial cell walls giving an appearance of scalariform xylem cells. 780 X. 11.) Multinucleate syncytial cells in sugar beet root; note the enlarged nuclei and nucleoli. 1720 X. 12.) Multinucleate syncytial cells in spinach. 1720 X.

the nucleoplasm stains a dark green. The cytoplasm becomes granular. Similar enlargement of nuclei and nucleoli, granularity in cytoplasm, and dissolution of cell walls extends gradually outward from the point of origin of the syncytium. The syncytium enlarges more rapidly longitudinally than radially. This change does not occur laterally or posteriorly to the nematode's body. Understandably, the cell walls of the developing syncytium are irregular and incomplete. The hypertrophied nuclei are confined by the cell wall fragments; this individuality of many syncytial cells is retained past the egg-laying stage of the female. Most frequently the syncytial cells are uninucleate, but multinucleate cells are also found (Figure 11). This condition is also found in other hosts, such as spinach (Figure 12).

The multinucleate condition in syncytial cells might arise by mitotic division or by pooling of nuclei from adjacent cells. In *Nacobbus*-induced syncytia, the individuality of many syncytial cells is visible and a multinucleate condition does not arise by pooling of nuclei. In sugar beet root galls, nucleoli are found budding with concomitant invagination of the nuclei (Figure 13A, B). This appears to be amitotic rather than mitotic division. Although hundreds of sections have been examined, mitotic figures have not been observed in syncytial areas. In *Kochia scoparia* and *Opuntia tortispina* root galls, budding nuclei are found which are abnormally shaped and somewhat cylindrical (Figure 14). This may be interpreted as disintegration of nuclei, except that this does not explain the multinucleate cells. Although it is the consensus of current investigators that the multinucleate condition in *Meloidogyne* and *Heterodera* infected tissues results from the pooling of nuclei from coalescing cells, it appears that in *N. batatiformis*-induced syncytia, the increase in number of nuclei is due to amitotic division.

The syncytium acts as a unit; cytoplasmic contents of the syncytial cells differ from adjacent normal cells. In *Opuntia tortispina,* druses (composed of calcium oxalate) are absent in the syncytia, but present in the adjacent normal cells. Starch induction in the syncytium is apparent in root galls of sugar beets and other hosts (5). Starch is a very early histological symptom occurring with the initial hypertrophy of cells and nuclei (Figure 15). The starch grains at this early stage of infection stain faintly with crystal violet and are smaller than in later stages of infection. The starch grains appear first in the periphery of the nuclei (Figures 7, 13A, 16). As the syncytium increases in size, starch is conspicuous near the feeding area of



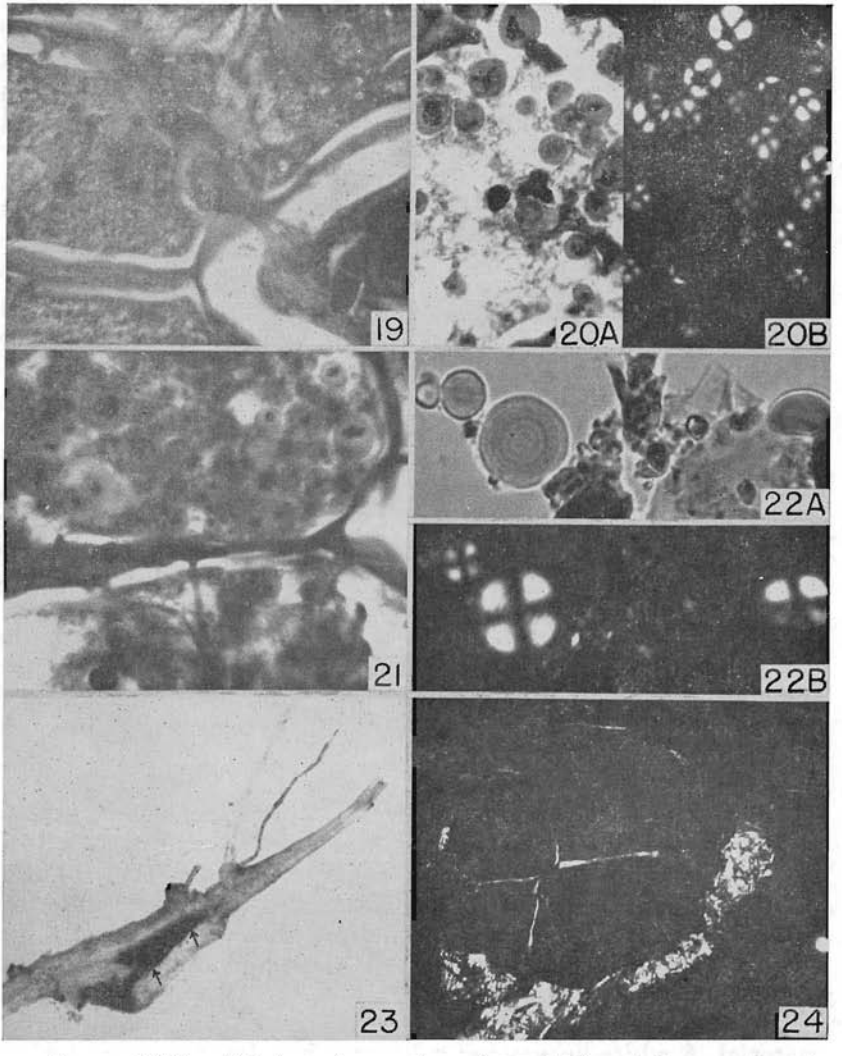
Figures 13-18.—13A.) Nucleolus budding with concomitant invagination of the nucleus in syncytium of sugar beet gall. 1100 X. 13B.) Similar condition in syncytium of spinach and the resulting multinucleate condition due to amitotic divisions. 1100 X. 14.) Abnormal cylindrical nuclei in syncytium in *Kochia* showing densely stained areas. The long axes of the nuclei are usually parallel to that of the root. 3500 X. 15.) Syncytium in 5-day old gall showing presence of small starch granules occurring with the initial hypertrophy of cells, nuclei, and nucleoli; arrows point to starch grains and transverse section of young larva. 970 X. 16.) Starch grains are initiated in vicinity of the nuclei of affected cells; similar condition is illustrated in Figures 7 and 13A. 800 X. 17.) Starch located near feeding area of adult female; starch granules are darkly stained bodies. 200 X. 18.) Starch granules are numerous during dissolution of syncytial cell walls. 750 X.

the nematode (Figure 17) but absent in the areas posterior or lateral to the parasite. This is substantial evidence that the nematode is directly responsible for starch induction due to its feeding processes. Starch grains are large and numerous during dissolution of synctial cell walls (Figures 18, 21). In advanced stages of infection when the female is in the process of egg-laying, there is either a cessation of starch synthesis or starch carbohydrate may be utilized in reproduction (Figure 19). In tissue culture, males have been found to induce starch but because of their mobility, it is difficult to determine if starch is utilized.

Starch grains are spherical and range in size from 1-30 μ in diameter in sugar beets (Figure 20A, B) and other hosts, such as *Portulaca oleracea* (Figure 21). The starch granules do not differ in optical properties from those that occur naturally in other plants. They are larger than those found in the sugar beet leaves in which the granules are about 0.1 μ. The symmetrical granules in the root galls have conspicuous concentric layering around a hilum when observed through oil immersion under bright light (Figure 22A) or polarized light (Figure 22B). Birefringent crosses typical of starch grains are noted when viewed through crossed polaroids.

General Considerations

It has been postulated that the giant cell complex caused by *Meloidogyne* spp. acts as a nectary in providing nutriments for the parasite. Initially the parasite modifies the host tissues by physical and chemical means. Similarly, *N. batatiformis* alters the metabolism of the cells of the syncytium to induce starch formation in relatively large quantities. The galls or the syncytia are nectaries or metabolic sinks upsetting the normal gradients in a curious manner. The gravid *N. batatiformis* female, by inducing hypertrophy and hyperplasia of cortical parenchyma, probably forces the plant to translocate a larger portion of plant nutrients to this area and thus insures the permanently located female a lasting nutrient supply. Not only does it appear that nutrients are differentially directed to the gall, but wound reaction has been instigated and wound phelloderm formation is in progress. The presence of starch in the syncytium is unequivocal evidence that the nematode incites a chemical change in the gall. This fact, the progressive dissolution of cell walls, hypertrophy of cells, nuclei, nucleoli, increase in granularity of the cytoplasm, and necrosis suggests a strong enzymatic activity of a type foreign to a normal root and therefore arising from the nematode.



Figures 19-24.—19.) Anterior portion of gravid female in syncytium in which starch is absent; note the channel in syncytium through which the nematode may obtain its nutrients. 750 X. 20A.) Induced starch grains are spherical and range in size from 1-30 μ in sugar beet galls; photographed with bright light. 720 X. 20B.) Same as 20A photographed with polarized light. 720 X. 21.) Starch grains in syncytium of *Portulaca oleracea*. 1200 X. 22A.) Starch grains in sugar beet gall showing concentric layering around a hilum photographed with bright light. 1250 X. 22B.) Same as 22A photographed with polarized light; note birefringent crosses typical of starch grains and concentric layering. 1250 X. 23.) Longitudinal section of iodine-stained sugar beet gall showing presence of starch as depicted by dark staining area in the syncytium as shown by arrows. 25 X. 24.) Giant cells caused by *Meloidogyne incognita* show no starch grains photographed with polarized light. 450 X.

The use of iodine staining might be a useful diagnostic method for the detection of *N. batatiformis* in sugar beet roots (Figure 23). Since none of the other gall-forming nematodes (Figure 24) cause starch formation, the iodine stain of a dissected gall may provide a quick method of identification. Care must be exercised in selecting fairly young galls since starch is often depleted in older galls. Usually all stages of gall and nematode development can be found during the growing season.

Fluorescence studies indicate that the changes in cells affected by *N. batatiformis* cause the cell walls to fluoresce similarly to epidermal walls. Epidermal walls and wound phellogen develop a layer of suberin. This coloration may indicate that the plant tissues react to the nematode as they would to mechanical wounding, by producing suberin deposits on the walls of the cavity cells. This would also explain the characteristic staining with safranin of these walls, for epidermal cells exhibit this type of reaction which is also true for necrotic cell walls and walls of cells surrounding the nematode. Comparable studies with *M. incognita* infected tissues show that these same staining reactions (bright light or fluorescent) do not occur. Since suberin is impervious to water, its deposition would interfere with the nematode's ability to obtain nutrients. *Nacobbus* ruptures cell walls, but it is not known whether this occurs before or after suberin deposition.

The coiled larval stages fluoresce a glowing white, mostly obscuring their interior structures. Young larvae exhibit a yellowish fluorescence that does not entirely obscure the internal structures. Stained with acid fuchsin, the nematode fluoresces a cherry red. The stain is concentrated in the hypodermis and cuticle. The esophageal bulb is very dark and dense.

The female fluoresces yellow in unstained paraffin-embedded material. A white fluorescing substance in a layer or two of plant tissue cells surrounds the female and the canal that extends posterior from her body to the outside of the gall. This canal may be the path of entry or perhaps the nematode exudes some substance that dissolves a channel preparatory to egg-laying. This channel is quite straight and narrow. The degree of fluorescence in the areas around the female and in the channel are about the same, indicating that the channel has been there for some time. The unstained areas that fluoresce cannot be distinguished from other adjacent cells when viewed through bright light. Thus, ultraviolet miscroscopy can be a tool to detect differences not observable through light microscopy.

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