Accumulation of Phytoferritin and Starch Granules in Developing Nodules of Soybean Roots Infected with *Heterodera glycines*

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ABSTRACT

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Structural changes in developing soybean (*Glycine max*) nodules as affected by race 1 of the soybean cyst nematode, *Heterodera glycines*, were investigated by light and electron microscopy. Emerging nodules from cyst-nematode-infected roots were poorly organized, with less distinct zones of nodular tissues and early appearance of vascular elements and sclerenchyma layers. However, the most conspicuous features in the infected plants were the massive accumulation of starch granules and crystalline arrays of phytoferritin in the plastids of cells in the nodular central tissues. Although small starch granules occasionally occurred in similar tissues of control plants, phytoferritin was not observed. Starch and phytoferritin are, respectively, energy and iron reserves. Their accumulation in nodules of nematode-infected soybeans suggests that the metabolism of carbohydrates and iron-containing compounds is affected by the presence of the cyst nematode.

Additional key words: nitrogen fixation, nodulation, *Rhizobium japonicum*.

The success of soybean nodule development requires a balanced supply of a wide variety of nutrients and growth factors (14) from the host plant and/or the rhizobia. The presence of a pathogen in a soybean plant may upset this balance and cause developmental and structural changes in the nodules. In fact, nodules of soybeans infected with the soybean cyst nematode (SCN), *Heterodera glycines* (7,12), and certain viruses (20) are fewer in number, smaller in size, lower in leghemoglobin content, or generally less effective in fixing nitrogen.

Our preliminary histopathological studies of nodules from control and SCN-infected soybeans with light microscopy indicated that structural developments in these nodules were apparently similar during the first 3 wk of nodulation (10); however, the effects of SCN on the fine structure of these nodules have not been examined previously. Suppressed nodule formation and limited nitrogen-fixing capacity, characteristically associated with SCN infection, strongly suggest that nodular development is affected by the nematode (1).

The objective of this research was to compare electron microscopically the structural changes occurring in nodular tissues of control and SCN-infected soybean plants. Particular attention was focused on the central tissues of young nodules because the early events there may be among the most susceptible to disruption by SCN during nodular development (10).

MATERIALS AND METHODS

Seeds of soybean, *Glycine max* (L.) Merr. 'Lee 68', were surface sterilized in a mixture of ethanol, commercial bleach (containing 5.25% sodium hypochlorite), and water (1:2:3, v/v) for 3 min. After several rinses in water, the seeds were germinated in vermiculite at 25 C for 5 days. Seedlings were transplanted individually into 10-cm-diameter clay pots containing white quartz sand (212 μm [65-mesh]) and inoculated with 10^8 colony-forming units of *Rhizobium japonicum* (Kirchner) Buchanan (strain 61A76). At the same time, half of the seedlings was inoculated with 5,000 SCN eggs; the other half was left uninoculated to serve as controls. Cultures of *R. japonicum* and SCN eggs were obtained and prepared as previously described (11). Plants were maintained in a greenhouse with supplemental light under a 16-hr light period at 29 $\pm$ 3 C and an 8-hr dark period at 24 $\pm$ 2 C. They were watered twice daily and fed with Evan's nitrogen-deficient nutrient (16) three times weekly.

Under these conditions, visible nodules appeared on the control soybean roots 9 days after inoculation, but were not observed on the SCN-infected plants until 21 days after inoculation. Nodules (0.5–1.0 mm in diameter) were harvested from control and SCN-infected plants 1–2 days after their emergence. At least 10 nodules were harvested from five different plants of each treatment. Nodules were fixed in 0.05 M potassium phosphate buffer (pH 6.8) containing 4% glutaraldehyde at room temperature for 4 hr., rinsed in phosphate buffer, and postfixed in 2% OsO_4 in the phosphate buffer for another 4 hr. Samples were washed in the same buffer, dehydrated in an alcohol series, infiltrated with propylene oxide, embedded in LX-112, and cured at 65 C for 72 hr. Sections, 1 μm thick, were cut with glass knives, placed on microscope slides, stained with toluidine blue (0.5% in 1% borax or 0.05% in 0.05M potassium phosphate buffer [pH 6.8]), and then examined under light microscopy. Ultrathin sections were obtained with a diamond knife, collected on 50 μm (300-mesh) copper grids, and stained as previously described (8). Unstained sections were also examined for the presence and measurement of phytoferritin. Electron micrographs were made with a JEOL 100 S electron microscope operated at 80 kV.

RESULTS

Nodules were first observed on roots of control soybeans 9 days after rhizobial inoculation, but these did not appear on the SCN-infected soybean roots until 12 days later (Fig. 1). Nodules on the SCN-infected plants grew at a much slower rate and there were fewer of them than on the controls. The few nodules that did

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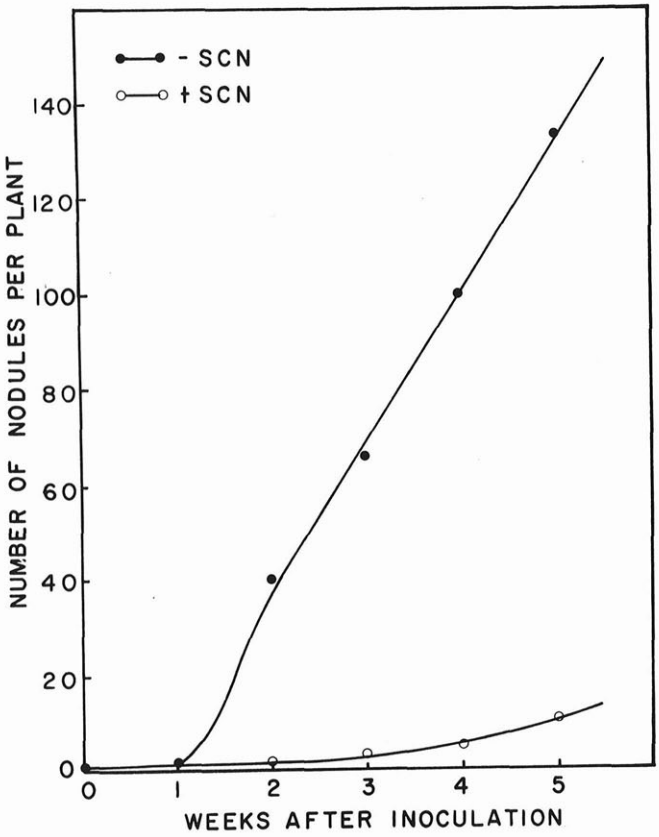
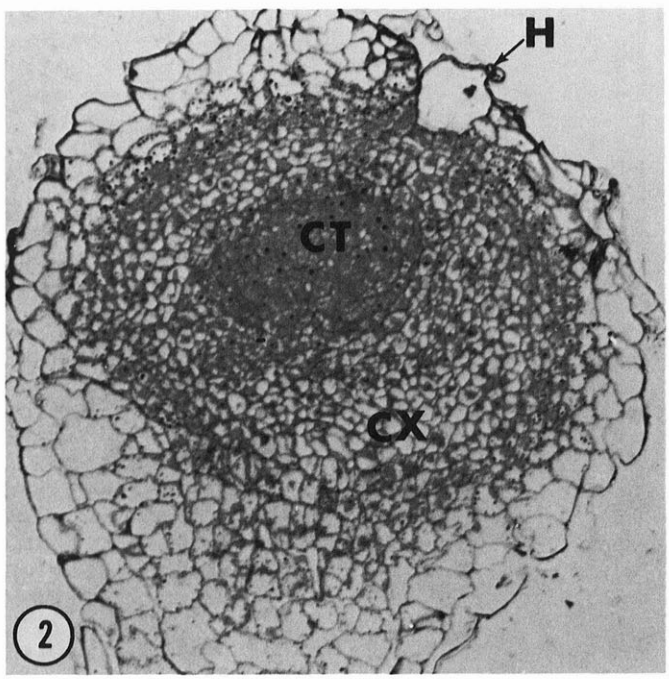


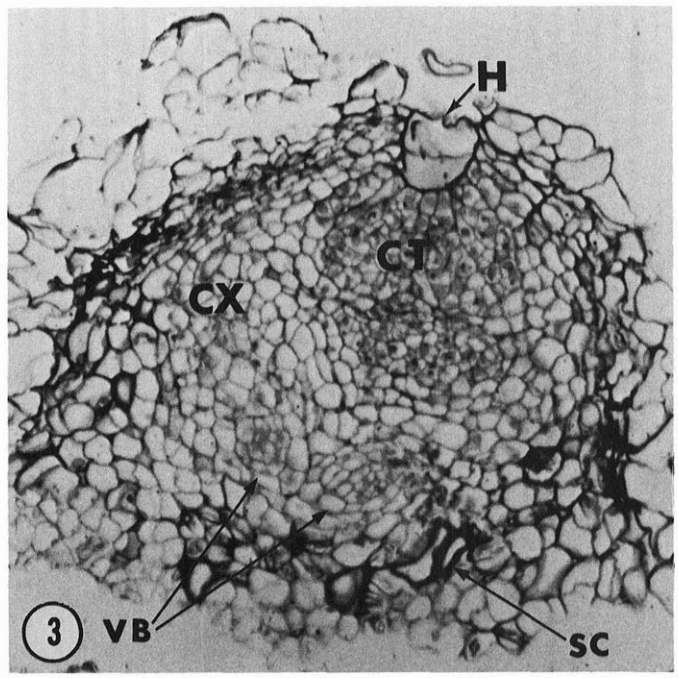
Fig. 1. Time course of the appearance of soybean nodules as affected by the soybean cyst nematode, *Heterodera glycines*, race 1 (SCN). Each plant was inoculated with either 0 (−SCN) or 5,000 (+SCN) nematode eggs. The mean number of nodules was calculated from 10 seedlings.

emerge from SCN-infected plants were darker in color and had hardened outer surfaces. No SCN juveniles were observed to be penetrating these nodules. Roots of the infected plants were discolored due to lesions caused by the nematodes; however, the size of the root system was not visibly affected by the nematode at this stage.

Examination of thick sections (1.0 μm) of emerging nodules (12 days old) from control and SCN-infected plants indicated that cellular differentiation had already begun, especially in the latter (Figs. 2 and 3). The meristems of nodules from control or SCN-infected plants had given rise to a peripheral uninfected tissue of cortical parenchyma cells, and a central tissue invaded by the bacteria (Fig. 2). Most of the cortical cells in nodules from control plants were still dividing, whereas some of those from SCN-infected plants had already differentiated into vascular elements. Although the basic cellular arrangement seemed to be similar between these two types of nodules under light microscopy, there were several noteworthy differences. The emerging nodules from control plants appeared to be more organized, with a central tissue developing evenly in all directions (Fig. 2). Nodule development from SCN-infected plants was more or less disoriented; thick-walled sclerenchyma cells appeared early around the cortex and the central tissue, particularly at base of the nodule (Fig. 3). The volume of enlarged infected cells in the nodular central tissues was also smaller than that of similar cells in the control.

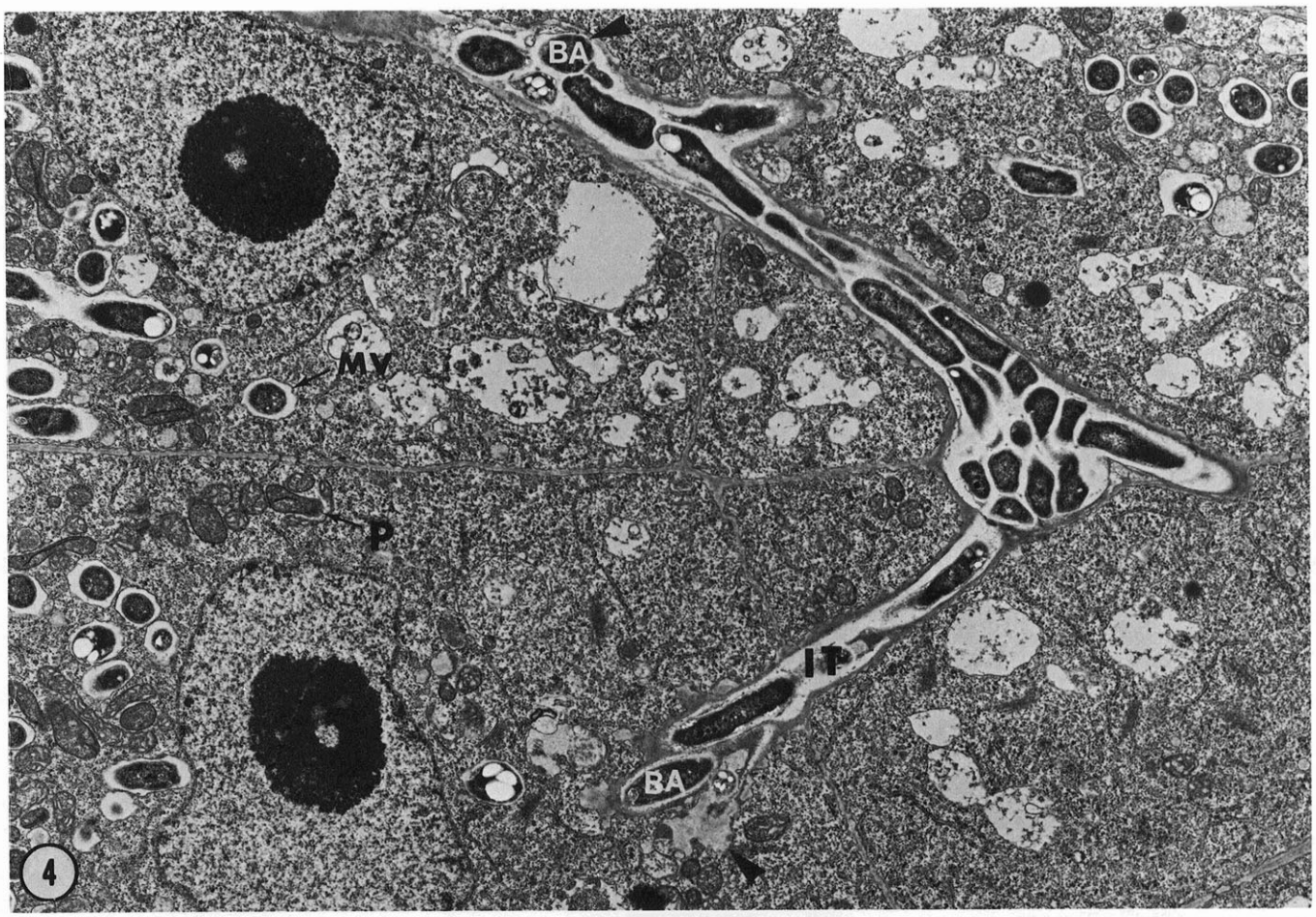
Electron microscopy of ultrathin sections of the nodular central tissues indicated that infection by *Rhizobium* proceeded similarly in both types of nodules. However, there were certain subtle differences. Infection threads ramified extensively in the central tissues of emerging nodules from control plants, with rhizobia actively multiplying inside. Release of rhizobia from the infection threads into the cellular cytoplasm in the nodular central tissue was frequently observed (Fig. 4). Localized dissolved areas were evident in the walls of the infection threads as the bacteria "budded off" individually in membrane vesicles from the infection thread. At this stage, the released rhizobia had not yet differentiated into swollen





Figs. 2–3. General organization of soybean nodules. **2,** Two-day-old nodule from a control plant. (×160). **3,** Nodule of same age from a soybean cyst nematode-infected plant. CX, cortex; CT, central tissue; H, hypertrophied root-hair cell; SC, sclerenchyma cell; VB, developing vascular bundle. (×160).

Figs. 4–5. Central nodular tissues in soybean. **4,** Tissues from control plant. An infection thread (IT) with dissolved walls (large arrows) for the release of rhizobia (BA) is evident. The released bacteria are enclosed in membrane envelopes (MV). No dense phytoferritin particle is apparent in the plastids (P) (×6,700). **5,** Tissues from a soybean cyst nematode-infected plant. Some degenerating bacteria (DB) are embedded in a thick matrix of mucilage (MU) within the infection thread (IT). Numerous plastids (P) with prominent inclusion bodies are present. (×10,000).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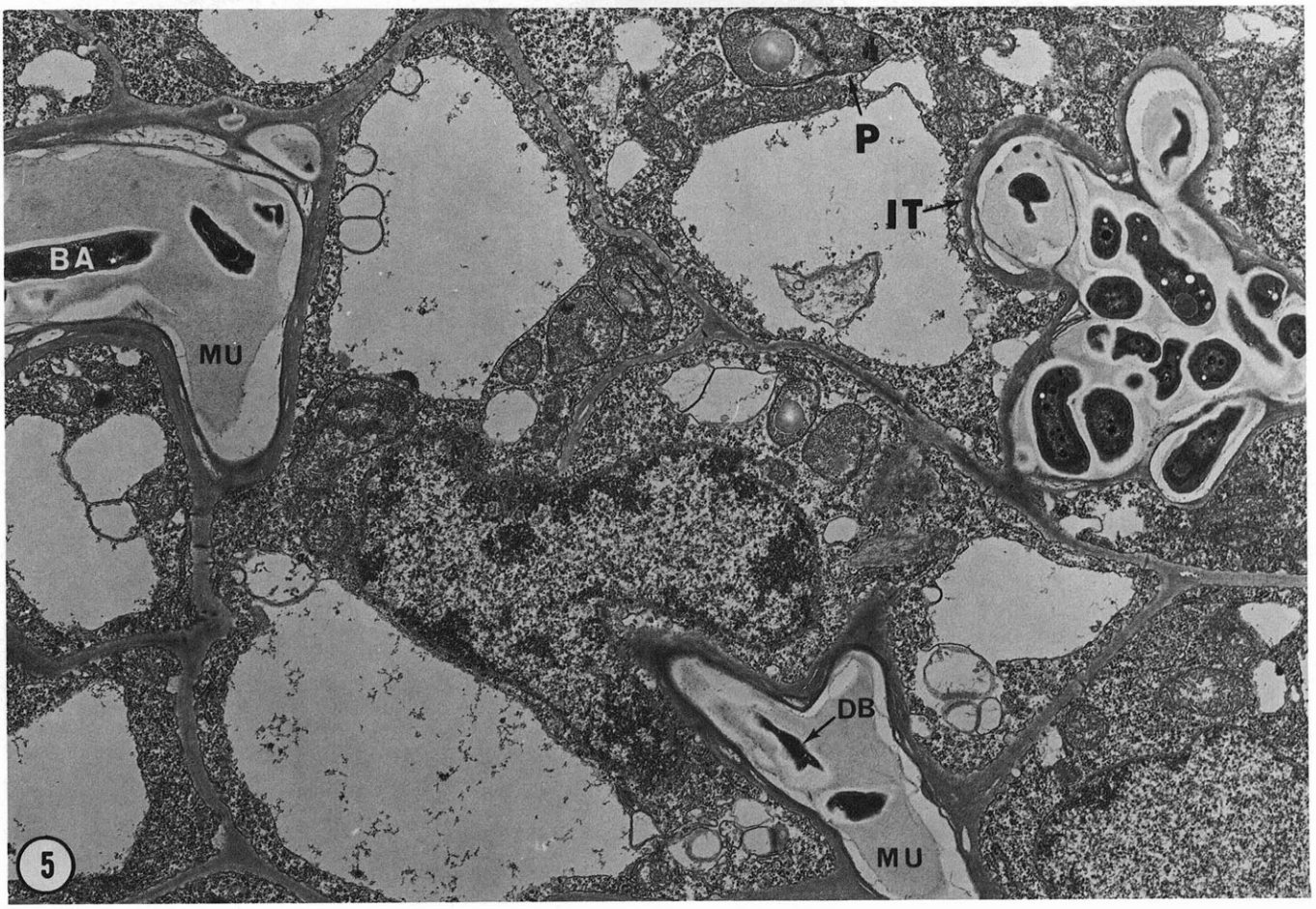
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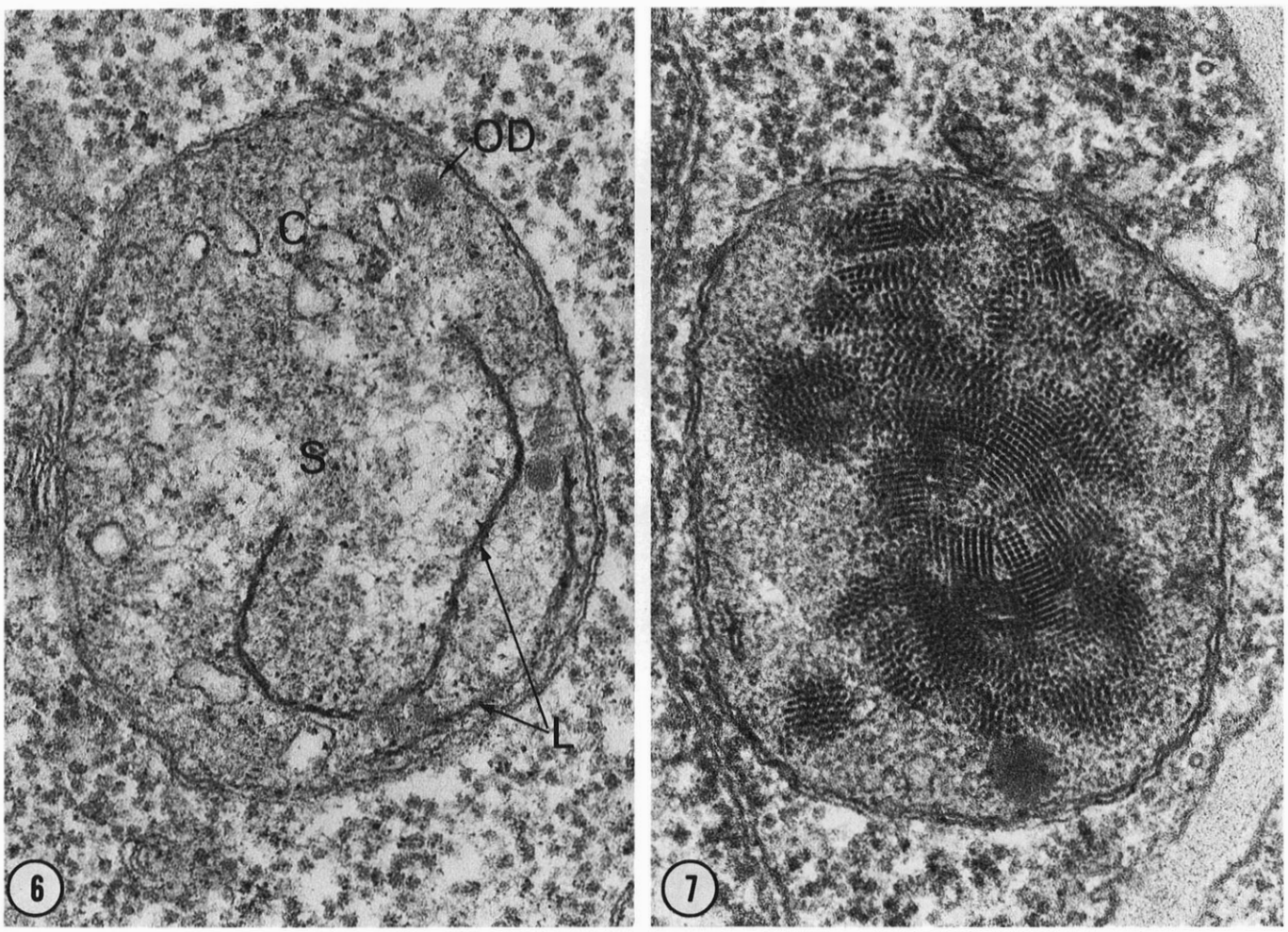
bacteroids. In similar nodular tissues of the SCN-infected soybeans, infection threads generally had large empty spaces, fewer rhizobia, and compact walls with no sign of weakening in any area. Many rhizobia in these infection threads were embedded in a thick matrix of mucilage or polysaccharide and appeared shrivelled, losing their structural integrity (Fig. 5).

Numerous plastids of various sizes and shapes were observed in cells in the nodular central tissues of control soybean plants (Figs. 4 and 6). In addition to the occasional occurrence of small starch granules, lamellae, cisternae, and osmiophilic globules were observed in the stroma of these plastids; no dense phytoferritin particles were apparent. In contrast, the plastids in the nodular tissues of SCN-infected plants were conspicuous because of the massive accumulation of starch granules and crytalline arrays of phytoferritin in their stromas (Figs. 7 to 10). The occurrence of these starch and phytoferritin bodies was most abundant in nodules with thick-walled sclerenchyma cells. The starch granules were spherical to ovoid and were either deposited between, or partially surrounded by, the lamellae (Fig. 9). Although phytoferritin was most frequently associated with starch granules, arrays were occasionally found alone in plastids (Fig. 7); these dense inclusion bodies did not seem to have a particular localization within a plastid, but large clusters were often observed at the end of an elongated plastid. The phytoferritin particles were also seen in ultrathin sections without uranyl and lead staining (Fig. 10). Individual particles on unstained sections were about 6.0 nm in diameter, and the center-to-center distance between the particles was approximately 11.8 nm. Particles on stained sections had an average diameter of 10.5 nm. These dimensions were very similar to those reported for animal and plant ferritins (4,9).

DISCUSSION

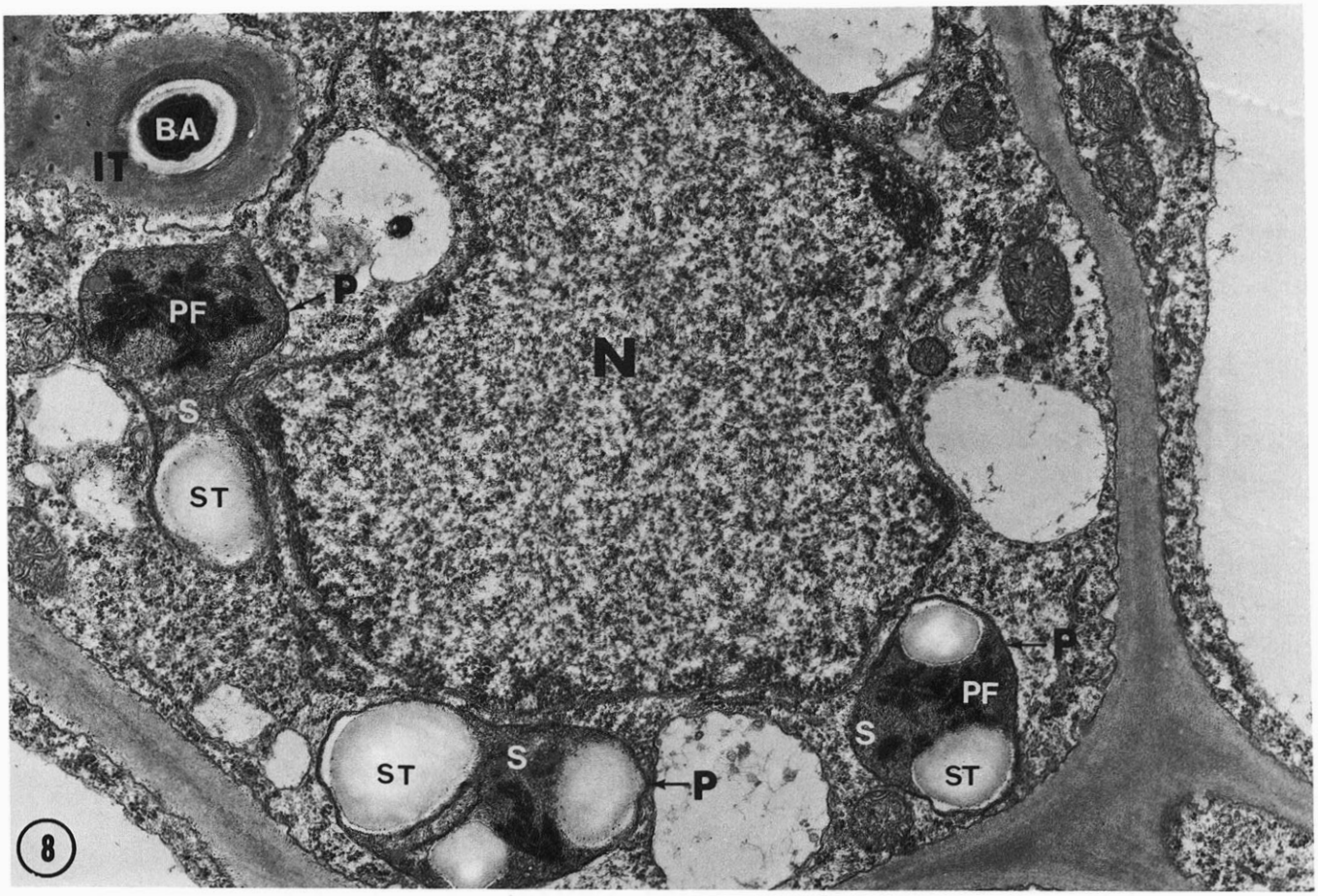
Nodulation and nitrogen fixation in soybean involve many components in a delicately balanced state. Studies have indicated that a genetic defect in either partner, rhizobia or legume host, would result in the arrest of one or more nodule developmental sequences (21). Biotic and abiotic stresses on either partner would also disrupt the nodulation process (14). Experiments on rhizobia-cyst nematode-soybean interactions reported in this paper exemplify the interference of a pathogenic nematode in the soybean-rhizobia symbiosis.

The slow rate of nodule appearance and nodule development in SCN-infected soybeans (Fig. 1) may be related to the lack of certain nutrients or growth factors to sustain rhizobial multiplication in the infection threads of nodules from diseased plants. Nematodes



Figs. 6–7. Young plastids from central nodular tissues in soybean. **6,** A plastid from control plant showing its stroma (S) containing osmiophilic droplets (OD), cisternae (C), and lamellae (L). (×77,000). **7,** A plastid from a soybean cyst nematode-infected plant showing its stroma largely occupied by prominent crystalline arrays of phytoferritin. (×77,000).

Figs. 8–10. Cellular structures of central nodular tissue from a soybean cyst nematode-infected plant. **8,** A cell showing various sizes and shapes of the plastids (P). Phytoferritin (PF) and starch bodies (ST) accumulated in the stroma (S) of the plastids, one of which is in the vicinity of the nucleus (N). One bacterium (BA) is surrounded by a compact-walled infection thread (IT). (×16,000). **9,** A typical plastid showing crystalline deposits of phytoferritin (PF) and an ovoid starch body (ST). A lamella (L) arising from the inner plastid membrane forms a partial loop around the starch body. (×64,000). **10,** (inset) An unstained ultrathin section showing regular patterns of crystalline arrays of phytoferritin. (×200,000).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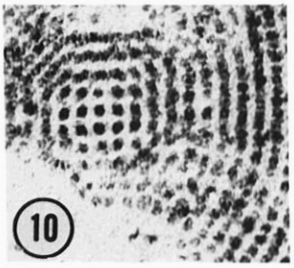
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are known to be powerful nutrient sinks and may therefore deprive the infected plant or/and rhzobia of many essential nutrients or growth factors.

Truchet et al (19) gave evidence that differentiation of nodular central tissues required the presence of rhizobia in the cytoplasm. Therefore, the slow growth of nodules in the SCN-infected plant may also be due to the subsequent lack of differentiation and enlargement of the larger number of uninvaded cells present in nodule initials, due to rhizobia not being released into the cytoplasm. Rhizobia are freed from the infection threads by disintegration of thread walls and endocytosis of bacteria by the host cytoplasm (2). The compact walls of infection threads and the associated thick polysaccharide-like matrix in nodules from SCN-infected tissues (Fig. 5) may serve as physical or antigenic barriers against the release of rhizobia into the host cellular cytoplasm or transport of nutrients from the cytoplasm into the infection threads. Polysaccharide, implicated in many cases to be involved in recognition (15), seemed to be more than normally associated with degenerating rhizobia in infection threads of ineffective nodules on alfalfa (18), or with released rhizobia that failed to develop into bacteroids in alfalfa nodules (21). Polysaccharide may play a certain role in altering the effectiveness of symbiotic associations.

The early appearance of thick-walled sclerenchyma cells and multiple meristemic sites has been observed with other ineffective legume nodules (5,19), as was observed in our experiment with nodules from SCN-infected plants. These responses may be attributed to hormonal imbalance, or to the physiological age of the nodules from SCN-infected plants. Although harvested nodules from control and SCN-infected plants were of comparable size and age after emergence, the latter were processed 10–12 days later. Therefore, it is not certain whether the nodules from SCN-infected plants were the newly initiated nodules, or the earlier ones that developed at a reduced rate. The differentiated morphology and the presence of thick-walled sclerenchyma cells in these nodules (Fig. 3) suggest that the latter may be true.

The most conspicuous ultrastructural changes associated with SCN infection were the massive accumulation of starch and phytoferritin in the plastids of cells in the nodular central tissues (Figs. 7 to 10). Starch granules are usually found in similar abundance in cells of many ineffective legume nodules, or in uninvaded cortical cells of developing effective nodules (5). In effective nodules, however, starch seldom accumulates in nodular central tissues where the cells are invaded by the bacteria (5).

Phytoferritin is an iron-storage protein similar to the ferritin found in mammalian cells, where it readily takes up excess iron entering the cytoplasm and rapidly releases it when needed for synthesis of iron-containing cellular constituents (17). It has been either observed in, or isolated from, plastids of different plant tissues, particularly leaves under iron overload and tissues not associated with active photosynthesis such as root tips, shoot and legume nodule meristems, and leaf cells either etiolated or yellowed by disease or senesence (4). The phytoferritin from the nodular tissue of the SCN-infected plants has the same particle sizes as that found in other plant tissues, and similarly, it is arranged in characteristic crystalline patterns in the stroma of plastids. Crystalline arrays of phytoferritin also are observed in plastids of certain ineffective legume nodules (5,18). In developing normal legume nodules, phytoferritin was observed as dispersed particles or dense aggregates (3,6), quite different from the crystalline form observed in this experiment. Although dispersed phytoferritin particles have not been observed in nodules from our control soybean plants, in disagreement with the report by Bergersen (3), the possibility that phytoferritin exists in these tissues as low-iron forms can not be ruled out. Van der Mark et al (13) showed recently with immunological techniques that phytoferritin existed in normal bean leaves in a form not demonstrable by electron microscopy, due to its low average iron content. Nevertheless, the fate and relationship of these ferritin types, and their role in the biosynthesis of the nitrogen fixation apparatus within the nodule should be further investigated because their disappearance in developing soybean nodules coincides with the appearance of leghemoglobin (3), and the leghemoglobin content in nodules from SCN-infected soybeans is severely reduced (7). Accumulation of starch and phytoferritin are often associated with immature or photosynthetically inactive tissues (4,9). Thus, it appears that nodules from SCN-infected plants were either relatively inactive metabolically or were under a prolonged period of immaturity that was not yet capable of utilizing the energy or iron reserves. Further studies are needed to determine whether this is the cause or effect of defective development of nodules.

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