Responses of Nodulation to Various Combinations of *Bradyrhizobium japonicum* Strains, Soybean Cultivars, and Races of *Heterodera glycines*

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ABSTRACT

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Nodulation efficiency of various combinations of bradyrhizobial strains, races of *Heterodera glycines*, the soybean cyst nematode (SCN), and soybean cultivars were investigated. Suppression of nodulation by SCN was influenced by the nematode race/soybean cultivar interaction but not by bradyrhizobial strains. Nodulation of susceptible Lee 68 soybeans was suppressed by race 1, regardless of the bacterial strain. In contrast, nodulation of resistant soybeans was less affected despite comparable penetration rates of juveniles and subsequent necrosis in the roots. Nodules from race 3-infected Lee 68 soybean had similar ultrastructures of bacteroid-containing cells and uninvaded cells and protein patterns in sodium dodecyl sulfate (SDS)-polyacrylamide gels, but reduced heme contents and nitrogen-fixation efficiency, as compared with those from the controls. Emerging nodules from race 1-infected plants accumulated phytoferritin and starch granules and had altered protein patterns in SDS-polyacrylamide gels, when compared to nodules of similar size from control or race 3-infected plants. Such differences were indicative of differentially altered nodule physiology and function as a result of infection by these two SCN races.

Additional keywords: *Glycine max*, host-parasite interaction, nematode races, nodule physiology.

The effects of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, on the *Bradyrhizobium*-soybean symbiosis are complex. Most studies involving this disease have been limited to a single soybean cultivar, *Bradyrhizobium japonicum* strain, and SCN race (2,8–10,17). Experiments with various combinations of susceptible or resistant soybean cultivars, *Bradyrhizobium* strains of varying symbiotic competence, and different SCN races have not been done. Such studies should facilitate identification of the roles of each component in these complex SCN-soybean-bacteria interactions.

Infection of soybean by a given *Bradyrhizobium* strain often varies under different environmental conditions (5,18). For example, invasiveness of bradyrhizobia was significantly reduced if the soybean plants were infected with viruses (21) or under nitrate stress (5). Thus, the symbiotic competence of bradyrhizobia is influenced by the changes in host physiology. The discovery and use of bacterial strains tolerant to SCN stress while maintaining nitrogen fixation has not been reported but would be of economic interest.

Soybean cultivars resistant to SCN typically develop a necrotic reaction upon penetration by SCN-juveniles (1,4,26). The accumulation of phytoalexin glyceollins around these necrotic sites has been noted (J.-S. Huang, *unpublished*). Although the sensitivity of bradyrhizobia to phytoalexins and isoflavonoids (22), and of other phytopathogenic bacteria, to glyceollins (6) has been reported, the impact of resistance responses induced by SCN infection on nodulation has not been thoroughly investigated.

Races 1, 2, and 4 of SCN cause suppression of nodulation and reduction of nitrogen-fixing capacity (17). Race 3, which occurs over the largest acreage in the United States (7), has not

been extensively investigated, although it is not thought to inhibit nodulation (25). Hypotheses have been offered to explain the various nodulation responses to different nematode races. These include differential rate of nematode penetration (16), selective inhibition of bacterial strains (16), and discrete competition for available carbohydrates or nutrients (12).

The objectives of this investigation were to determine: 1) the differential effect of SCN race 1 on nodulation by various bradyrhizobial strains; 2) the differences in nodulation responses among resistant and susceptible cultivars that are undergoing infection by race 1; and 3) the differential effects of races 1 and 3 on nodule formation and function.

MATERIALS AND METHODS

Biological materials. Soybean (*Glycine max* (L.) Merr.) cultivars Lee 68, Lee 74, Ransom, Centennial, Peking, and Pickett 71, all obtained from D. P. Schmitt of North Carolina State University (currently at University of Hawaii, Honolulu), were used in this investigation. *B. japonicum* strains 61A76 and 61A24 were obtained from Nitragin Company, Milwaukee, WI, and strains USDA 76, 94, 110, 122, 123, 135, and 138 and strain I110ARS (14) were obtained from the USDA collection at Beltsville, MD. These bacterial strains were maintained on yeast extract-mannitol (YEM) agar slants and cultured in YEM broth (27) just before use. Races 1 and 3 of SCN, originally from Wilmington, NC, and Tennessee, respectively, were maintained on Lee 68 soybeans grown in silica sand in the greenhouse.

Treatment and maintenance of soybean plants. Germination of seeds, preparation of bacterial and nematode inocula, and growth conditions of the soybean were as described (12,13). One-week-old seedlings were transplanted individually into 10-cm-diameter clay pots containing 212-μm white quartz sand and

inoculated with 10^8 colony-forming units of bradyrhizobia and 0 or 5,000 eggs of race 1 or race 3. Plants were harvested at various time intervals and nodules were immediately processed for electron microscopy (13), gel electrophoresis, or for protein and heme assays (11,13). Nodules not analyzed immediately were frozen at -80 C for later use. In some experiments, growth parameters such as shoot and root fresh weights, leaf protein, chlorophyll content, and nodulation parameters such as nodule number and weight, and nitrogen-fixing capacity were also measured at time of harvest. The nitrogen-fixing capacities of nodules in the excised roots were determined by their ability to reduce acetylene to ethylene (12).

The development of SCN in roots was monitored by harvesting samples (five replicates) from all treatments as described (12). The treatments within each experiment were completely randomized with at least five replications per treatment. Each experiment was performed at least twice.

Polyacrylamide gel electrophoresis. Nodule proteins were separated by SDS-polyacrylamide gel electrophoresis, with the discontinuous buffer system of Laemmli (15). Nodule proteins were solubilized in 10 mM Tris HCl, pH 8.0, that contained 1 mM EDTA, 0.25 M sucrose, 1% SDS, and 5% β-mercaptoethanol. Samples with equal protein concentrations were boiled for 5 min and applied to the sample wells. Electrophoresis was carried out at a constant current of 3 mA per gel. Gels were stained in a solution containing 0.05% Coomassie blue (w/v), 25% isopropyl alcohol (v/v), and 10% glacial acetic acid (v/v) and destained in a solution of 10% isopropyl alcohol in 10% glacial acetic acid.
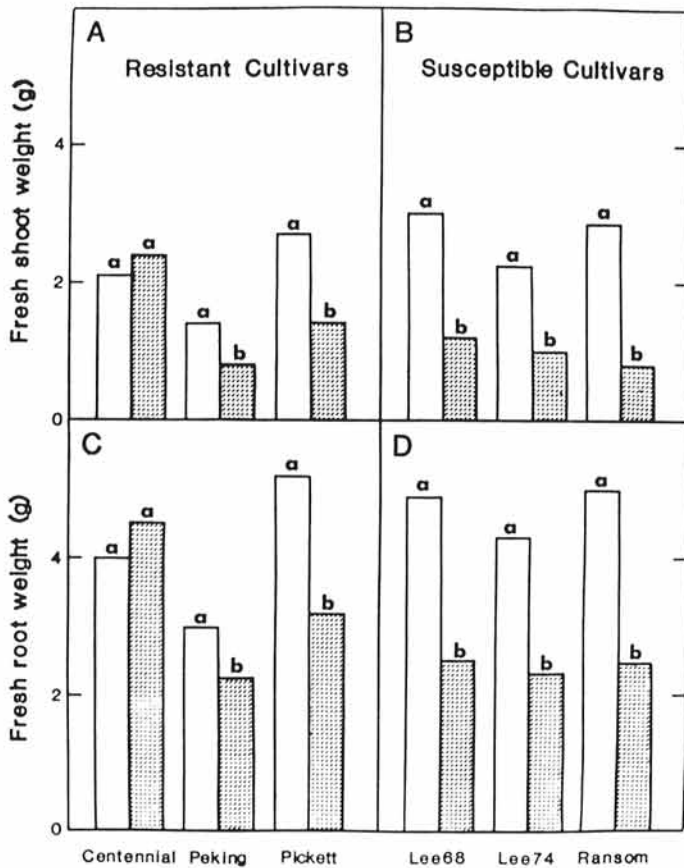
Gels were stored in 10% glacial acetic acid if not photographed immediately.

Statistical design and analysis. All data were subjected to analysis of variance, and Waller-Duncan mean comparisons were performed if the F value was significant.
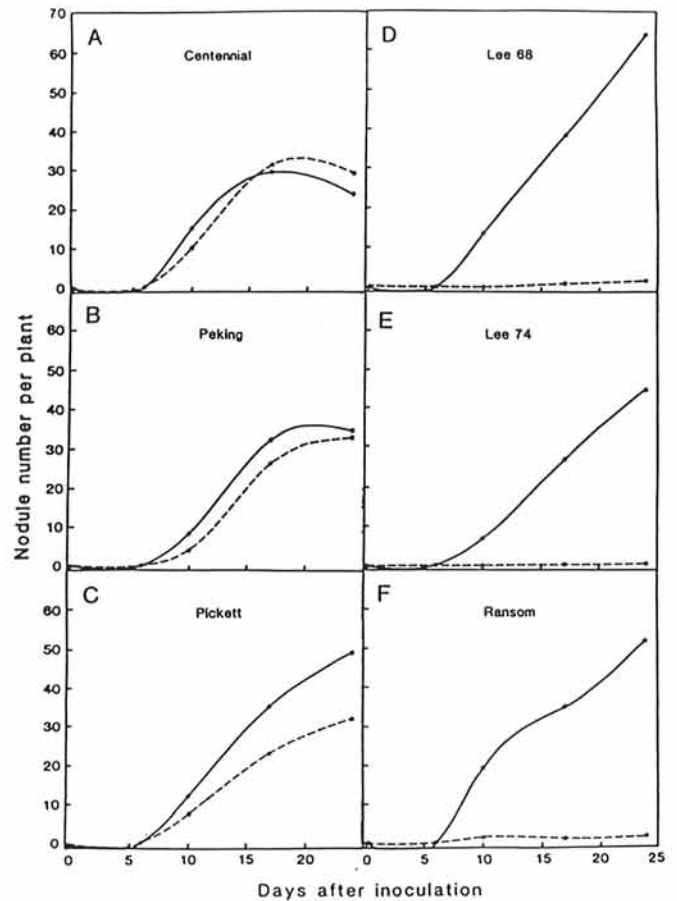
Fig. 2. Effect of race 1 of the soybean cyst nematode (SCN) on nodule number per plant of susceptible and resistant cultivars. The soybean plants were inoculated with *Bradyrhizobium japonicum* strain 61A76. **A–C,** Resistant cultivars. **D–F,** Susceptible cultivars. ——, not inoculated with SCN; - - - -, inoculated with SCN.

Fig. 1. Growth parameters of susceptible and resistant soybean cultivars as affected by race 1 of the soybean cyst nematode (SCN), 25 days after inoculation. The soybean plants were inoculated with *Bradyrhizobium japonicum* strain 61A76 with (filled bars) or without (open bars) 5,000 eggs of SCN race 1. **A and B,** fresh shoot weight (g/plant) of resistant (A) and susceptible (B) cultivars. **C and D,** fresh root weight (g/plant) of resistant (C) and susceptible (D) cultivars. The data are means of five replicates. Means with the same letter in the same cultivar are not significantly different ($P = 0.05$) according to the Waller k-ratio t test.
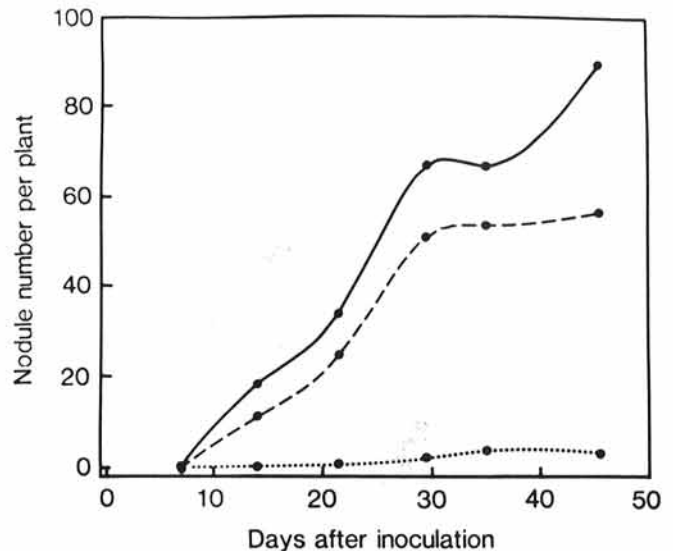
Fig. 3. Effects of races 1 and 3 of *Heterodera glycines*, the soybean cyst nematode (SCN), on nodule number on Lee 68 soybean. The soybean plants were inoculated with *Bradyrhizobium japonicum* strain 61A76 and 0 (——), 5,000 eggs of SCN race 1 ($\cdots$), or race 3 (- - - -).

Nodulation of SCN race 1-stressed Lee 68 soybean plants by Bradyrhizobium strains. In the absence of SCN race 1, all *Bradyrhizobium* strains used in the experiments varied in nodulation efficiency and promotion of shoot and root growth. None of the strains were able to form nodules in the presence of race 1 when examined 25 days after inoculation. Approximately 31–48% of the 5,000 eggs inoculated to each seedling hatched and penetrated soybean roots and of these 23–50% eventually developed into mature cysts. None of the bacterial strains exerted significant influence on cyst development.

Nodulation responses of susceptible and resistant soybean cultivars under SCN race 1 stress. Shoot and root growth of susceptible cultivars (Lee 68, Lee 74, Ransom) were severely inhibited when measured at 25 days after SCN inoculation, compared to their respective controls (Fig. 1B and D). SCN also reduced root and shoot growth in seedlings of resistant cultivars Peking and Pickett; the growth of resistant cultivar Centennial, however, was not affected by SCN (Fig. 1A and C). Nodulation by *B. japonicum* strain 61A76 was almost totally suppressed in susceptible cultivars (Fig. 2D–F) but only slightly affected in resistant cultivars (Fig. 2A–C). Nitrogen-fixing capacity (μmoles C_2H_4 reduced/plant/hr) and nitrogen-fixing efficiency (μmoles C_2H_4 reduced/g nodule/hr) of nodules from SCN-infected resistant plants at 4 wk after inoculation were lower than those from the control plants, but differences were not significant ($P = 0.05$).

Similar numbers of SCN-juveniles penetrated roots of resistant and susceptible cultivars. At 6 days after inoculation with 5,000 eggs of SCN race 1, 15–18% of the eggs hatched and the juveniles penetrated roots of susceptible soybeans as compared with 17–21% penetrated roots of resistant cultivars. The percentages that eventually developed into cysts in resistant and susceptible plants, however, were different. At 25 days after inoculation, 24% of the juveniles in roots of susceptible cultivars had developed into adult females, but in resistant cultivars, only 0.06–0.12% of the juveniles had similar development. Examination of roots at week 3 indicated that most of the juveniles in resistant cultivars did not develop beyond the third stage, but 612–668 pear-shaped females per root were observed in susceptible cultivars. Necrotic lesions were present around the infection sites of resistant cultivars.

Effects of SCN races 1 and 3 on nodulation and ultrastructure of nodular tissues of Lee 68 soybean. Seedlings infected with SCN race 1 were stunted and had few nodules (Fig. 3) and low nodule weight. The seedlings infected with race 3 were less affected (Fig. 3). There was no significant difference ($P = 0.05$) between the rates of penetration by races 1 and 3 and their subsequent development into cysts. At 8 days after inoculation, average of 798 juveniles of race 1 and 773 juveniles of race 3 were found in each root of inoculated Lee 68 soybean seedlings. Approximately 47 and 54% of the penetrated race 1 and race 3 juveniles, respectively, developed into mature females at 28 days after inoculation.

Although appearance of the first nodule in race 1-infected seedlings was delayed or sometimes completely suppressed, appearance (with respect to that of control seedlings) in race 3-infected plants was not affected. Examination of emerging nodules <1.5 mm in diameter from race 3-infected and control seedlings at 10–12 days after inoculation showed that the ultrastructures of bacteroid-containing cells and uninvaded cells were very similar. Compared with the control plants (Fig. 4A), starch granules were more abundant in the nodular tissues from race 3-infected seedlings (Fig. 4B), reflecting the change in physiology of the N_2-fixing apparatus in these nodules. Phytoferritin, which occurred in more than 50% of plastids in nodules from race 1-infected seedlings (Fig. 4C), was observed in less than 1% of plastids in nodules from race 3-infected seedlings. No phytoferritin was observed in nodules from control soybean (Fig. 4A). The premature appearance of sclerenchyma cells and disorganized meristems, characteristic of emerging nodules from race 1-stressed plants, also were not noticeable in nodules from race 3-infected soybean

plants. More than 50% of nodules from control and race 3-infected plants were greater than 1.5 mm in diameter at 5 wk after inoculation. In contrast, fewer than 5% of the nodules from race 1-infected plants were greater than 1.5 mm in diameter. SDS-gel electrophoresis of nodule proteins prepared from 5-wk-old nodules of all the treatments indicated that protein patterns of nodules from control and race 3-infected plants were generally similar at this stage of development (data not shown). However, both qualitative and quantitative changes in certain protein bands occurred in samples prepared from nodules >1.5 mm and <1.5 mm in diameter of race 1-infected plants (data not shown).

DISCUSSION

Nodulation of soybean by various *Bradyrhizobium* strains was abolished by race 1 of SCN. The result implies that this race interferes with some nodulation mechanism common to all these strains. It is known that infection of soybean roots by bradyrhizobia and the subsequent development of nodules are similar (19,24). These common nodulation steps (3,30) are disrupted by SCN. Although none of *Bradyrhizobium* strains tested were able to form nodules on Lee 68 infected with high levels of SCN, some strains did exhibit nodulating ability at low SCN infection rate (2,12). Inoculation with high doses of *B. japonicum* increases nodule formation in soybeans (18). Thus, differential tolerance of *Bradyrhizobium* strains to nematode stress is more apparent at lower SCN levels.

The rate of nodule development in resistant soybean plants under SCN stress was relatively unaffected by race 1 infection despite the penetration of large numbers of SCN juveniles into the roots. Rapid root necrosis is known to occur in both susceptible and resistant associations but more frequently in the resistant ones (1,4,26). These necrotic reactions and other putative resistance responses, such as phytoalexin accumulation, to SCN in roots of resistant cultivars seem to have only a minor effect on nodulation during the early stages of soybean growth. Our results (Fig. 3) also indicated that the presence of SCN-induced syncytia was not solely responsible for nodulation suppression, since syncytia were present in race 3-infected susceptible cultivars, yet considerable nodulation of these tissues occurred.

Although nodulation in race 3-infected plants was not affected as severely as in race 1-infected soybeans (Fig. 3), their heme contents and nitrogen-fixing capacity were markedly reduced at week 7 after nematode inoculation (data not shown). The decline in heme levels and a reduction in N_2-fixing capacity are indicators of premature nodule senescence (28). Causes for nodule dysfunction at week 7 observed on SCN-infected plants are complicated by secondary infection of root and nodular tissues by the progeny of the primary SCN inoculum (17). Direct effects on nodule function by the penetration of SCN juvenile into the nodular tissue have not been determined. The continued and increased stress on the host by the primary and secondary SCN inocula, and the direct attack of the nodule by juveniles may be responsible for the premature senescence of these nodules.

The abundant electron-dense phytoferritin particles observed in race 1-stressed nodules (Fig. 4C), but only rarely seen in race 3-stressed nodules, further indicate the specificity of the SCN race-soybean interaction. These observations indicate that SCN races affect not only the nodulation process but also nodule function and physiology as well.

Normal nodulation is subjected to both internal (23) and external regulation (18). Perhaps SCN race 1 elicits premature host regulatory suppressive responses over nodule formation and function more readily than race 3. Studies have shown that nodule formation is inhibited by the availability of nitrogen in the soil, by prior exposure of more mature regions of the primary root to bradyrhizobia, and by the emergence of the first nodule (20). The SCN race 1-soybean interactions are in many ways similar to these nodulation suppressive phenomena (18). Therefore, the use of SCN race-soybean cultivar combinations that allow differential nodulation may provide additional systems for studying the regulatory mechanism of nodulation. Studies of the
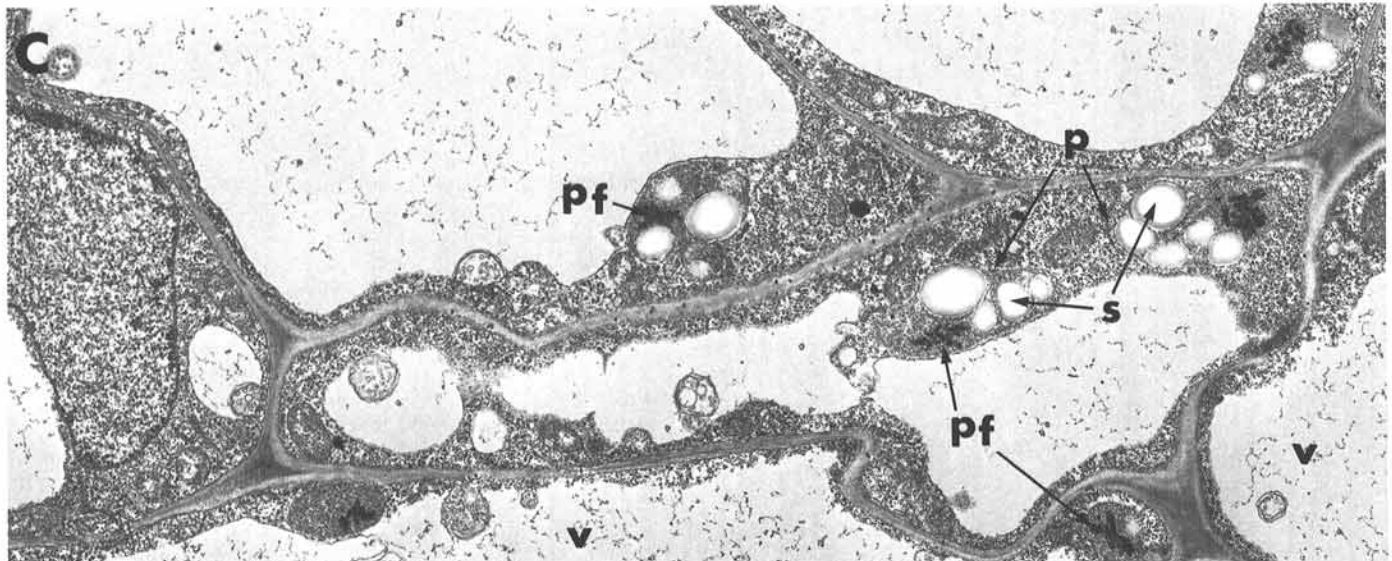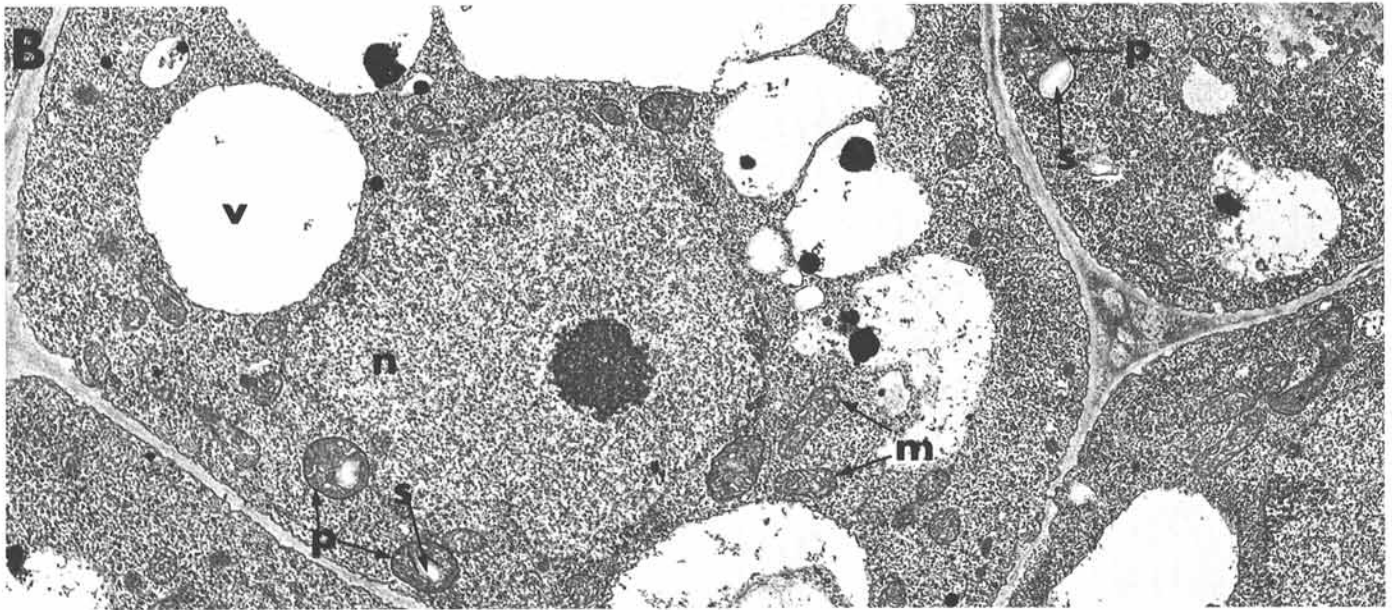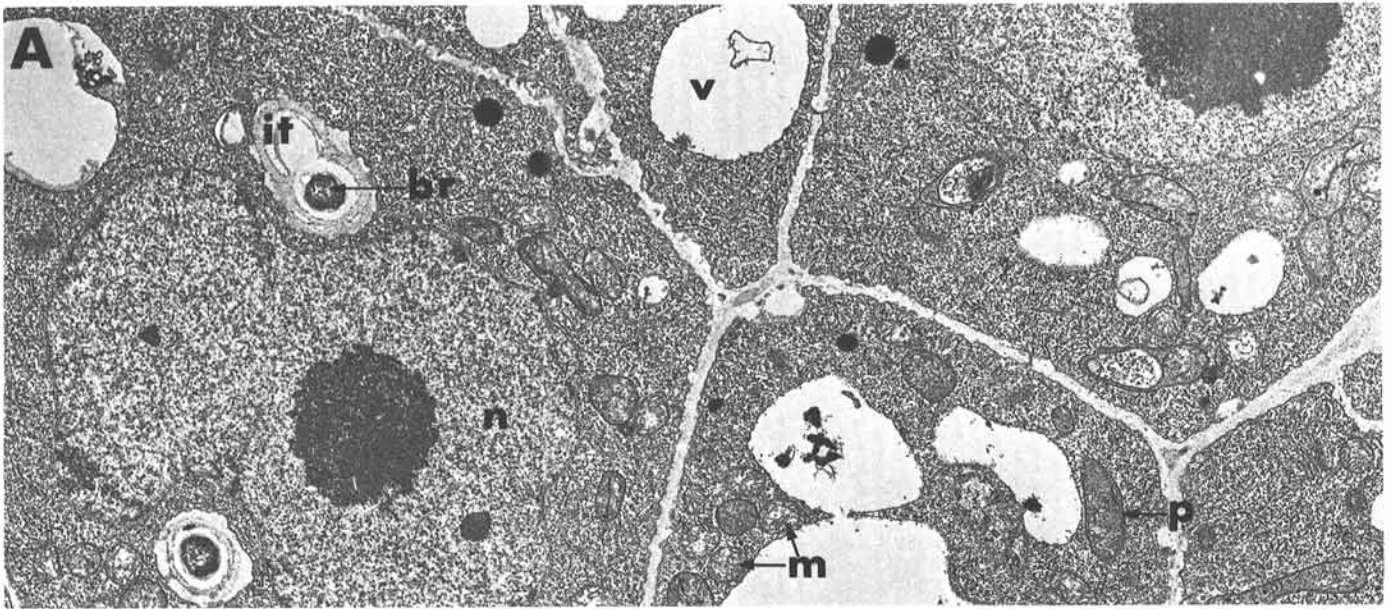
Fig. 4. Transmission electron microscopy of nodules from Lee 68 soybean, 10 days after inoculation with race 1 or 3 of *Heterodera glycines* and, *Bradyrhizobium japonicum* strain 61A76. **A,** Nodular central tissue from control plant (×7,800). **B,** Nodular central tissue from SCN-race 3 infected plant (×7,800). **C,** Nodular central tissue from SCN-race 1 infected plant (×7,800). br, *Bradyrhizobium* cell; it, infection thread; m, mitochondrion; n, nucleus; p, plastid; pf, phytoferritin; s, starch granules; v, vacuole.

nodulation-suppressive phenomena in these various systems will provide additional insight into the physiological, biochemical, and molecular mechanisms involved in the regulation of nodulation.

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