Interrelationships between Root-nodule Bacteria, Plant-parasitic Nematodes and their Leguminous Host[1]

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Abstract: The effect of infection by *Meloidogyne javanica* and *Heterodera trifolii* on number, size, structure and efficiency of nodules formed by *Rhizobium trifolii* on white clover roots was investigated. Introduction of nematodes one week before, simultaneously, or one week following inoculation with *Rhizobium* bacteria did not hinder nodule formation. Nodule size did not differ between nematode-infected and nematode-free plants. Formation of nodules on *M. javanica* galls and gall formation on the nodules have been reported. The structure of nodular tissues was not disturbed by nematode infection, even though giant cells were formed inside the vascular bundles. The nitrogen-fixation efficiency of nematode-infected nodules was not impaired; however, earlier disintegration of nodules as a result of *M. javanica* infection ultimately deprived the plants of nitrogenous materials. The drastic reduction of the total-N in *H. trifolii*-infected plants reflected stunting of the entire plant due to nematode infection. Both nematodes invaded the entire root system, uniformly showing preference for nodules.

Several workers have reported cyst and root-knot nematodes causing reduced nodulation on leguminous plants, e.g., *Heterodera glycines* Ichinohe on soybean (6, 7, 8, 25), *Heterodera trifolii* Goffart on white clover (27), *Heterodera goettingiana* Liebscher on peas (10, 20), *Meloidogyne* sp. on peanuts (15), and *Meloidogyne javanica* (Treub) on hairy vetch (12) and on alfalfa (16). *Meloidogyne hapla* Chitwood on hairy vetch (12) completely inhibited nodulation.

The cause of reduced nodulation is not known. According to Masefield (14), nematode galls on the roots may affect nodulation by causing nutrient deficiency in host plants and by occupying space on the root system, a reason which was supported later by Malek & Jenkins (12). A competition phenomenon between nematode larvae and root-nodule bacteria was also postulated as a cause of the reduction (6, 7, 12). An antagonistic effect of root-rot organisms upon root-nodule bacteria was also proposed as a possible cause of reduced nodulation (6).

Nodule invasion by *M. Javanica* has been reported in cowpea nodules (23) and in alfalfa nodules (16). Christie (4) also reported *Meloidogyne* sp. in soybean nodules. The effect upon nodular tissue of nematodes inside the nodules is not yet clear. Robinson (23) suggested that nodules attacked by nematodes during early stages of development become galls, but when attacked at a later stage they remain nodules.

Since the nodules are the centers of symbiotic nitrogen fixation, invading nematodes may disturb their function. It has been shown that the amount of nitrogen fixed by clover nodules is related to the total amount and longevity of the active nodular tissue before its disintegration (1).

The objectives of these studies were to explore causes of reduced nodulation in nematode-infected white clover, histological examination of nematode-infected nodules, and evaluation of nematode effects upon N-fixation.

Materials and Methods

All studies were carried out in a greenhouse at 24 C. The original inoculum of *Rhizobium trifolii* Dangeard was obtained

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from nodules of white clover plants in turf on the campus of the University of California, Davis. Cultures of *R. trifolii* were maintained in the greenhouse on Dutch white clover (*Trifolium repens* L.) grown in sterile washed sand and watered daily with nitrogen-free nutrient solution (22). The inoculum was prepared by grinding some nodules in a mortar with water to make a heavy suspension. Ten ml of suspension were added to each pot when required. No attempt was made to count number of rhizobia in the inocula because a surplus is usually present in the rhizosphere (19, 21).

Cultures of *M. javanica* and *H. trifolii* were maintained in the greenhouse on tomato plants (*Lycopersicon esculentum* L. var. Rutgers) and on Dutch white clover, respectively, growing in 15.2 cm pots in sterile washed sand. They were watered daily with tap water and with nutrient solution once a week.

Meloidogyne javanica egg masses were obtained from tomato roots by the technique of Lownsbery & Viglierchio (11) and placed in the mist chamber for 48 hr. At the end of the first day, the collected larvae were refrigerated at 6 C until the next day, when they were combined and used with the second collection.

Heterodera trifolii cysts were collected by wet-sieving, placed in a mist chamber and the larvae collected as described above for *M. javanica.*

The number of larvae per ml of suspension was determined by averaging the counts from 5 replicates of 1 ml each. Dilutions were made to obtain different levels of inoculum. Unless otherwise indicated, the inoculum was 1000 larvae per pot.

All experiments were replicated five times, using 30-day-old Dutch white clover seedlings for each treatment. The seedlings were grown singly in 10.1 cm pots or 15.2 cm pots containing washed sterile sand and watered with nutrient solution containing nitrogen (as calcium nitrate) (22). After inoculation with rhizobia, plants were watered with nitrogen-free nutrient solution throughout the experimental period.

Clover seedlings were inoculated with (i) rhizobia alone, (ii) rhizobia plus nematodes, (iii) rhizobia followed by nematodes after one week, or (iv) nematodes followed by rhizobia after one week. Nematode inocula were added to all treatments at the same time to minimize differences in nematode infectivity. In the third treatment, seedlings one week older were used in order to coincide with the timing of addition of nematodes to the other treatments.

For the effect of nematode infection on nodulation, the four treatments were carried out in 10.1 cm and 15.2 cm pots for 30-day and 77-day periods, respectively, from the time of the last addition of either rhizobia or nematodes.

For the effect of nematode infection on the total-N content, only the first and the third treatments were carried out in the same manner and for the same periods as above. During the 77-day period, two harvest cuttings were made: the first, 30 days after the time of nematode addition; the second, 30 days after the first. At the end of the 30-day and 77-day periods, plants were washed and the entire vegetative growth and roots were harvested.

Other replicate groups of the first and third treatments were left for 75 days without cutting. After this period, tops and roots of 3 replicates of each treatment were prepared for total-N determination. The other 2 replicates of each treatment were discarded after removing every nodule of each plant. The nodules were prepared for total-N determination.

One week after the soil was infected with rhizobia, 1000, 2000, 3000, 4000 and 5000 larvae were added. Plants inoculated with

rhizobia alone were maintained as a check. All plants were maintained for 30 days following the addition of nematodes.

Inoculation with rhizobia was made 7, 15 and 30 days after addition of nematodes. The same timings of rhizobial inoculation were followed with non-infected seedlings. All plants were maintained for one month after inoculation with rhizobia.

At the end of the experiments, roots were washed and weighed. Roots infected with *M. javanica* were stained with acid fuchsin in cold lacto-phenol and stored in it for not less than 24 hr. Stained roots were rinsed in water and cut into pieces to facilitate counting of galls and nodules. Roots infected with *H. trifolii* were not stained. Cysts and nodules were counted. Nodules infected with either species of nematode were counted separately.

Ten healthy nodules from each replicate of non-infected roots, ten infected nodules from each replicate of infected roots and ten nodules established on galls from each replicate were selected randomly. Length and width of each nodule were measured by using an ocular micrometer in the dissecting microscope.

Nodulated roots, tops and separate nodules were dried, weighed, ground, and analyzed for total-N by semi-micro Kjeldahl procedure. Ammonia obtained by steam distillation of the Kjeldahl digest was titrated with 0.505N H_2SO_4. Percent-N and total-N (mg) were calculated.

Healthy nodules and nodules infected with *M. javanica* or *H. trifolii* and also nodules developed on *M. javanica* galls were selected from 67-day-old and 107-day-old plants. Because of the degeneration of the nodules formed on galls at the age of 107 days, they were discarded.

Specimens were washed thoroughly with tap water and a fine brush to remove sand particles. Specimens were fixed in FAA at least 24 hr and serially dehydrated in ethyl alcohol. After clearing in xylene, they were embedded in paraffin, sectioned (7 μ) longitudinally and transversely, and stained with safranin-fast green (9).

TABLE 1. Average number of nodules, larvae inside nodules, Nod/Root ratios, and size of nodules on roots of white clover after various exposures to rhizobia and nematodes.

Treatment	Avg. dry wt. of roots (gm)	Avg. no. of nodules	Nod/Root (/1)	Larvae per nodule	Size of nodules (mm)
Rhizobia	1.773	853	500	–	5.69 × 2.25
Rhizobia + *M. javanica*	1.771	577	353	37	5.28 × 2.19
Rhizobia + *H. trifolii*	0.941*	439(*)	495	4	4.92 × 2.57
Rhizobia followed by *M. javanica*	1.951	770	423	39	5.31 × 2.73
Rhizobia followed by *H. trifolii*	1.060*	342**	322	10	5.61 × 3.15
M. javanica followed by rhizobia	1.748	689	445	27	5.22 × 1.91
H. trifolii followed by rhizobia	0.972*	364**	378	4	5.10 × 2.24

* Differs significantly from the other treatments at the 0.05 level.
** Differs significantly from the other treatments at the 0.01 level.
(*) Differs significantly only from rhizobia at the 0.05 level.

RESULTS AND DISCUSSION

Nematode infection did not significantly reduce the number of nodules per gram of root (Nod/Root ratio) or the size of nodules after 30 days or 77 days (Table 1) of nematode infection. Reduced numbers of nodules on *M. javanica*-infected plants (Table 2) and on *H. trifolii*-infected plants (Table 3) was due to overall reduction of the root system.

TABLE 2. Average numbers of galls and nodules and Nodule/Root ratios on roots of white clover grown with different numbers of *M. javanica*.

Number of larvae/pot	Avg. fresh wt. of roots (gm)	Avg. no. of galls	Avg. no. of nodules	Number of nodules/gm root (Nod/Root)
0	1.96[a](**)	–	366[a](**)	189
1000	0.98[b]	124	170[b]	177
2000	0.78[b]	199	115[b]	157
3000	0.58[c]	176	99[c]	179
4000	0.40[c]	117	50[c]	140
5000	0.58[c]	90	70[c]	125

Values not followed by the same letter differ significantly from one another at the 0.05 level, except asterisked values which differ significantly at the 0.01 level.

To further investigate effects of nematode infection on nodulation, inoculation with rhizobia was delayed to allow a large number of galls to develop (5, 17). The significant reduction in the number of nodules and Nod/Root ratio on plants heavily infected with *M. javanica* (Table 3) where rhizobia were added to 60-day-old plants was due to heavy nematode damage of the roots. An average of 15 nodules per gall developed on *M. javanica* galls, a situation which differs from Masefield's (14) conclusion that nodulation is prevented at sites occupied by nematode galls.

Increasing *H. trifolii* inoculum levels from 1000 to 5000 per pot or delaying introduction of rhizobia caused no significant difference in number of nodules, fresh weight of roots, or Nod/Root ratio even though cyst counts increased from 510 to 1826.

All nodules developed following delayed (30 day) rhizobial inoculation were infected by the added nematodes. *M. javanica* larvae per nodule ranged from 1–184, averaging 17, and *H. trifolii* larvae per nodule ranged from 2–23, averaging 6.

At 30 days the number of infected nodules was maximum when nematodes were added one week following introduction of rhizobia: *M. javanica* and *H. trifolii* averaged 68 and 15 per pot, respectively. When nematodes were added simultaneously with rhizobia, the number of nodules infected with *M. javanica* or *H. trifolii* was 14 and 3, respectively. The absence of nodules infected with either nematode when rhizobia were added after nematode inoculation was probably due to the prior entry of all larvae capable of causing infection.

After 77 days of nematode infection, 100% of the nodules selected at random from roots infected with *M. javanica* were infected (range 1–156 larvae/nodule), and 90% of the nodules from roots infected with *H. trifolii* were infected (range 1–29 larvae/nod-

TABLE 3. Average number of galls, nodules and Nod/Root ratios when rhizobia are added 7, 15, 30 days after addition of *M. javanica* larvae.

Age of plants when rhizobia added (days)	Avg. fresh wt. of roots (gm)		Avg. no. of galls		Avg. no. of nodules		Nod/Root (/1)	
	check	nematode added	check	nematode added	check	nematode added	check	nematode added
30 + 7	4.68	4.96	0	195	733	598	156	124
30 + 15	4.30	5.40	0	226	829	1320	195	249
30 + 30	6.42	9.06	0	769[a]	947	484	148	53*

* Differs significantly from its check at the 0.05 level.
[a] The average number of larvae collected by wet sieving was 359 × 10^2/pot.

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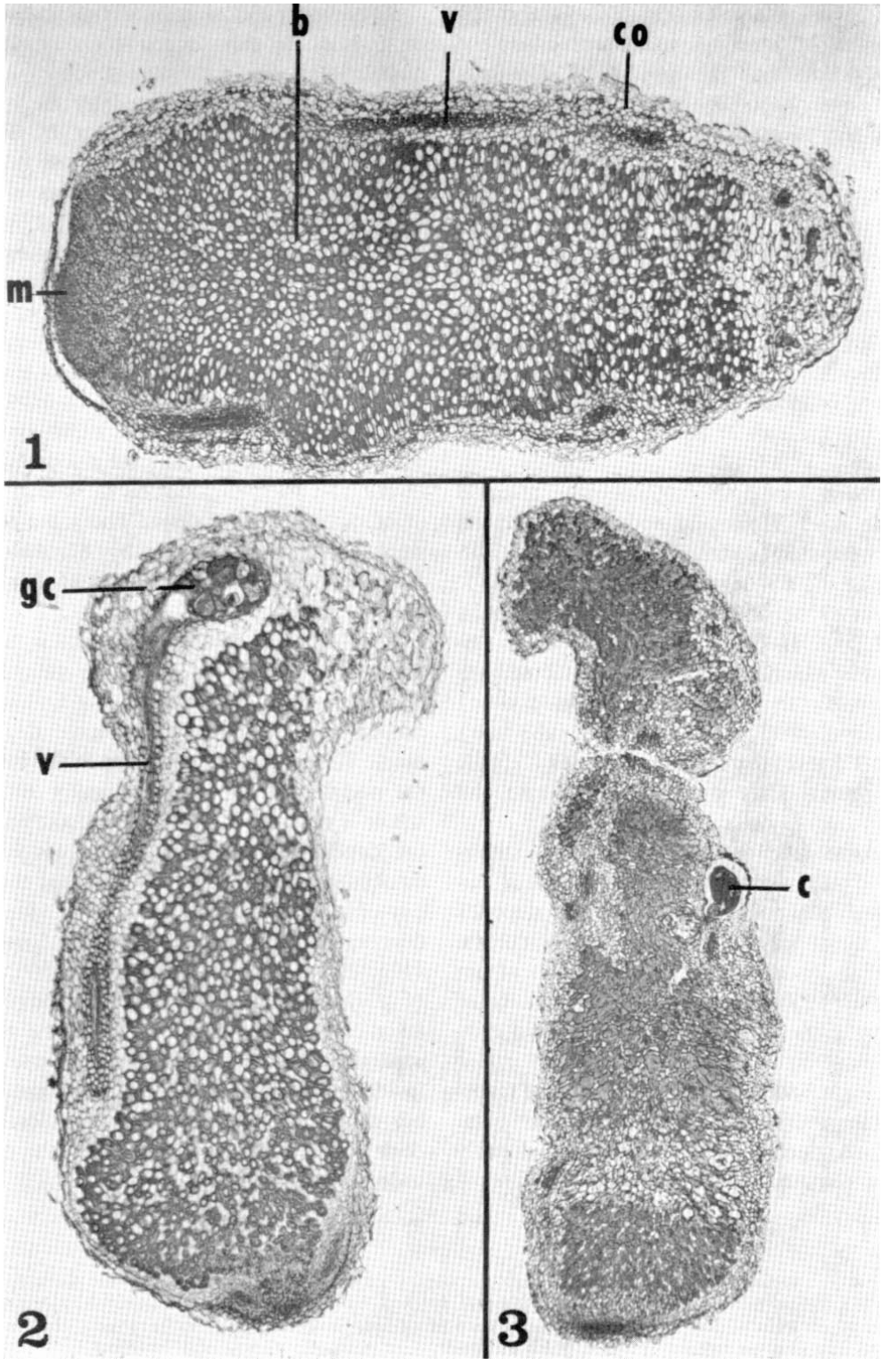
FIG. 1–3. 1. Longitudinal section of healthy nodule. 2. Longitudinal section of *M. javanica*-infected nodule showing the giant cells inside the vascular bundle. 3. Longitudinal section of *H. trifolii*-infected nodule showing the cyst extending to the cortex. (b=bacterial tissue; c=cyst; co=cortex; gc=giant cells; m=apical meristem; v=vascular bundle.)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ule). Swollen females (range 2–4) and egg masses of *M. javanica* were observed on the infected nodules. The number of *H. trifolii* cysts on each nodule ranged from 1 to 12. Secondary invasion of nodules by both nematodes was observed.

Data obtained provided no support for the hypothesis that nematode larvae reduce nodulation by competing with root nodule bacteria for root invasion sites (6, 7, 12). Nodule formation took place either before or after the addition of nematode larvae. However, nematode infection can affect number of nodules per plant indirectly by reducing the size of the root system (Table 3).

Neither nematode significantly affected nodule size. However, nodules formed on galls were significantly smaller (3.07 × 1.46 mm) at the 5% level than healthy (3.80 × 2.26 mm) or infected nodules not on galls (3.52 × 2.16 mm). Individual cases occurred in which nodules heavily infected with *H. trifolii* (an average of 6 cysts/nodule) were significantly smaller (0.98 × 0.83 mm) at the 1% level than healthy nodules (3.68 × 1.80 mm). They were soft in texture and dark brown in color.

Meristematic activity is doubtless stimulated by the bacteria (1), and from a nutritional viewpoint, nodule growth depends upon the supply and translocation of certain materials, particularly carbohydrates, from the top (19). Therefore, the occurrence of smaller sizes of nodules on nematode galls and those heavily infected with *H. trifolii* cysts is probably due to interruption of translocation and/or consumption of host plant nutrients during gall formation or directly by the nematodes in the case of *H. trifolii*.

Robinson (23) suggested the production of nodules or galls depends on how early nodulation begins following attack by nematodes. This is not supported by the results reported here. *M. javanica* or *H. trifolii* inside the nodule did not disturb or prevent the development of nodular tissues similar to those of healthy nodules (Fig. 1, 2, 3).

The presence of giant cells extending into the vascular bundles was observed in infection by both species of nematodes (Fig. 6, 7). The giant cells of *H. trifolii* (Fig. 6), also called syncytia by Mankau and Linford (13) differ from the giant cells of *M. javanica* (Fig. 7). The lateral walls of the syncytia of the former are broken and discontinuous, in contrast to the unbroken thickened walls of *M. javanica* giant cells. The same results were obtained previously by Mankau and Linford (13) for *H. trifolii* and *M. hapla* on Ladino clover root.

The life of an individual nodule is, unlike the root, generally of short duration (18). Therefore, it is not unusual to find a few non-infected nodules which have degenerated. Tissue breakdown starts at the base of the nodule and progresses apically until the whole is destroyed. Histological study shows the degeneration of nodular tissues of 77-day-old nodules (Fig. 9). Most of the 77-day-old nodules infected with *M. javanica* degenerated earlier than healthy nodules (Fig. 8) or nodules infected with *H. trifolii* (Fig. 10). The rapid degeneration of *M. javanica*-infected nodules cannot be attributed entirely to the presence of nematodes inside the nodules and/or to the large number of larvae reinfecting the nodules. The destruction of the root system by the nematode is also important.

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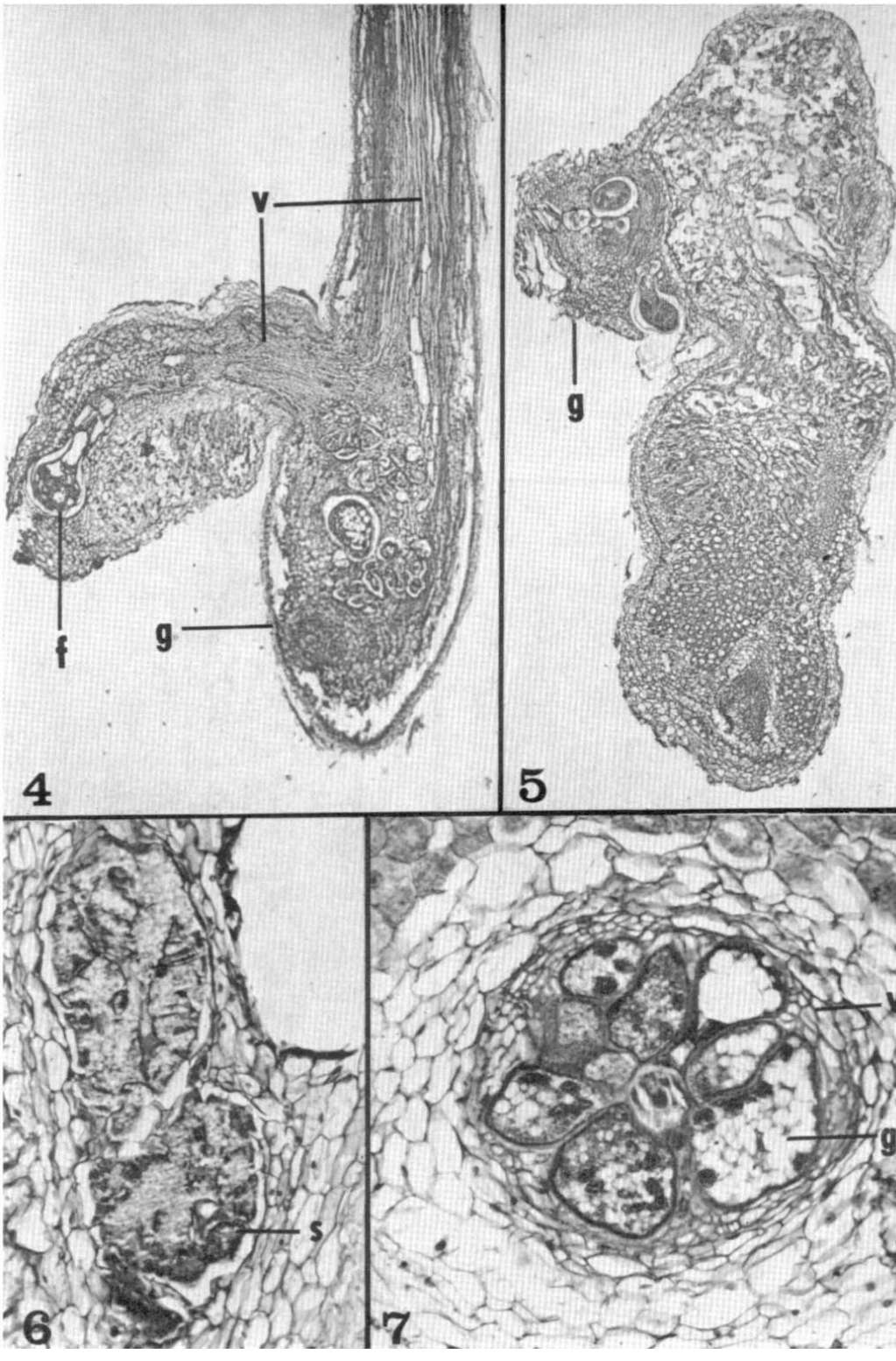
FIG. 4–7. 4. Longitudinal section through the cortex of *M. javanica*-infected nodule developed on gall. 5. Longitudinal section of nodule with gall formed by *M. javanica* infection. 6. Longitudinal section of *H. trifolii* giant cell. 7. Transverse section of *M. javanica* giant cells inside the vascular bundle. (f=swollen female; g=gall; gc=giant cells; s=syncytium; v=vascular bundle.)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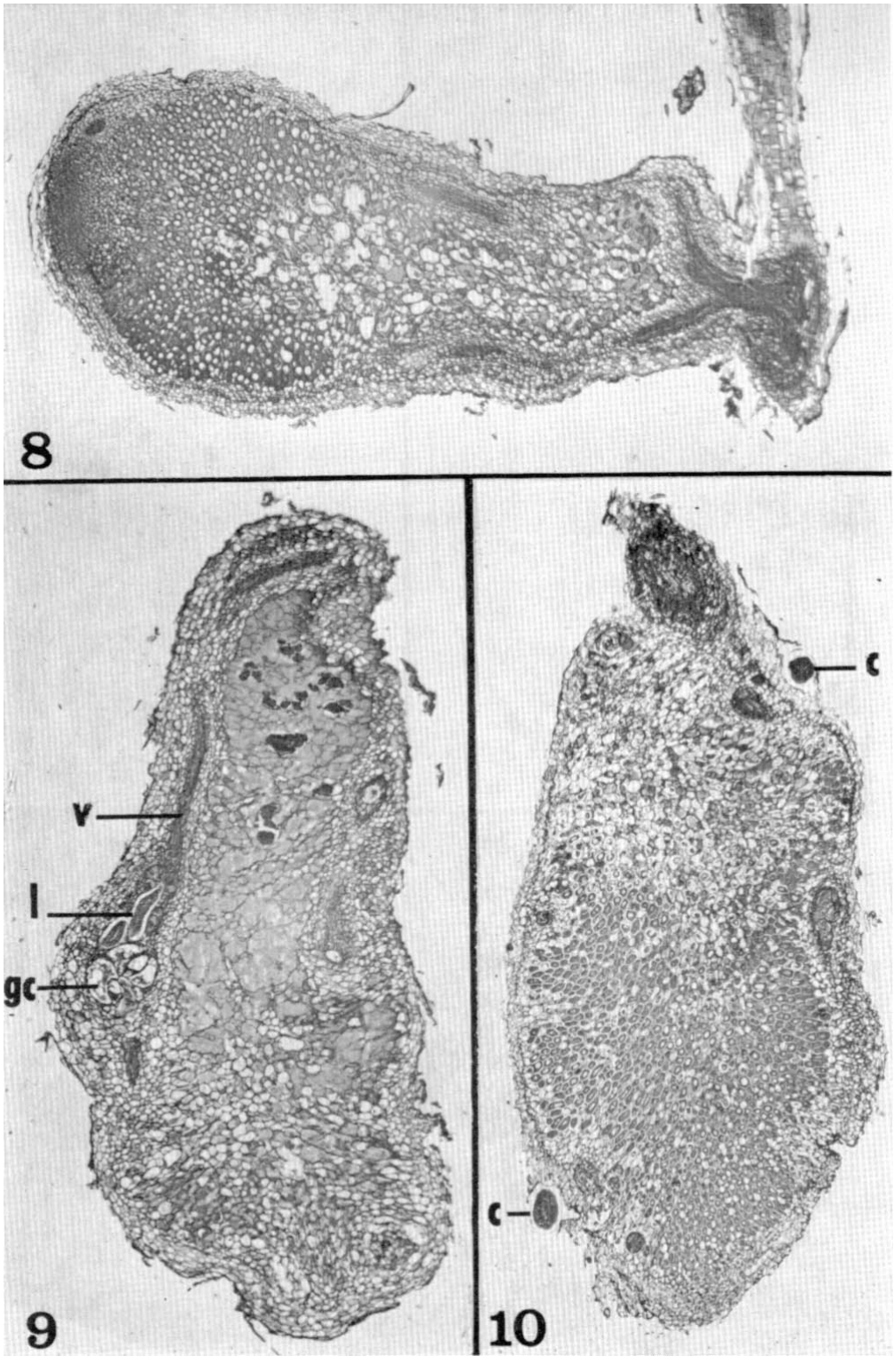
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TABLE 4. Total-N(%) and total-N(mg) of nematode-free plants and plants infected with *M. javanica* or *H. trifolii*.

Treatment	Dry wt. (gm) root	Dry wt. (gm) top	Total-N (%) root	Total-N (%) top	Total-N (mg) root + top
Rhizobia	0.399	0.898**	3.481	3.898	49.307**
Rhizobia followed by *M. javanica*	0.532	1.418	3.570	3.831	73.878
Rhizobia	0.289	0.677	3.464	4.320	39.519
Rhizobia followed by *H. trifolii*	0.392	0.826	3.437	4.277	49.093

** Differs significantly from *M. javanica*-infected treatment at the 0.01 level.

Histological observations confirmed the formation of nodules on *M. javanica* galls (Fig. 4) and also gall formation upon nodules (Fig. 5).

The addition of *M. javanica* or *H. trifolii* did not change the total-N(%) of tops and roots after 30 days of infection (Table 4 & 5). The significant increase of the total-N

TABLE 6. Dry weight, total-N(%) and total-N(mg) of nodulated plants and of nodules of non-infected plants and plants infected with *M. javanica*.

Treatment		Avg. Dry Weight (gm)	Avg. Total-N (%)	Avg. Total-N (mg)
Non-infected plants	tops	8.039*	3.613*	288.564*
	roots	2.023	4.072*	81.951*
	Total			370.515*
Infected plants	tops	3.950	2.881	111.752
	roots	1.429	2.021	38.258
	Total			150.010
Non-infected nodules		0.406*	6.415	25.655*
Infected nodules		0.181	6.182	11.308

* Differs significantly from infected treatment at the 0.05 level.

TABLE 5. Total-N(%), total-N(mg) and dry weight of tops and roots of different rhizobia and nematode infestation treatments during a 77-day period.

Treatment	1st Cutting dry wt. (gm)	1st Cutting total-N (%)	2nd Cutting dry wt. (gm)	2nd Cutting total-N (%)	Final Harvest tops dry wt. (gm)	Final Harvest tops total-N (%)	Final Harvest roots dry wt. (gm)	Final Harvest roots total-N (%)	Whole plant total-N (mg)
Rhizobia	1.341	4.419	1.943	4.899[a]	2.606[a]	3.928[a]	1.224	3.641[a]	302.533[a]
Rhizobia followed by *M. javanica*	1.609	4.117	1.961	4.358[b]	1.581[b]	2.734[d]	1.200	2.249[c]	221.509[b]
Rhizobia followed by *H. trifolii*	0.833	4.165	2.127	4.225[b]	1.153[b]	3.247[c]	0.847	3.611[a]	195.046[b]

In each column, means not followed by the same letter differ significantly from one another at the 0.05 level (a-b & c-d) and at the 0.01 level (a-c & a-d).

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FIG. 8–10. 8. Longitudinal section of 77-day old healthy nodule. The proximal half of the nodule is undergoing degeneration. 9. Longitudinal section of 77-day-old nodule infected with *M. javanica* undergoing almost complete degeneration of bacterial tissue. 10. Longitudinal section of 77-day-old nodule infected with *H. trifolii* showing the same degree of degeneration of that of Fig. 8. (c=cyst; gc=giant cells; l= larvae; v=vascular bundle.)

(mg) fixed in the case of *M. javanica*-infected plants (Table 4) results from the greater dry weight of the tops. This dry weight increase might reflect stimulation of plant growth by nematode infection (2, 3, 26). The average number of *M. javanica* inside the roots was 124 per plant.

The effect of nematode infection on total-N(%) appeared after 60 days of infection and continued to increase with both nematodes, especially so with *M. javanica* (Table 5). Chen & Thornton (1) named two quantitative characters closely correlated with effectiveness of nodules: the combined volume of the nodular tissues and the length of time that elapses before they collapse and disintegrate. Similar conclusions may be drawn from data presented in Table 6. The total-N(%) of healthy nodules and nodules infected with *M. javanica* (Table 6) was the same. This indicates that nematode infection probably did not hamper the efficiency with which bacterial cells fix nitrogen. But early destruction of nodules as a result of *M. javanica* infection deprived the plants of some nitrogenous materials. Romaniko (24) reported the same condition caused by *Pratylenchus globulicola* Romaniko in peas, alfalfa and clover nodules.

The significant reduction of the total-N (mg) of *H. trifolii*-infected plants (Table 5) resulted from the lower weights of the entire plants caused by nematode infection.

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