Interaction of Acrobeloides buetschlii and Rhizobium leguminosarum on Wando Pea

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ABSTRACT

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Invasion by and reproduction of *Acrobeloides buetschlii,* a microbivorous nematode, inhibited N_2 fixation in nodular tissue of Wando pea, *Pisum sativum*. High numbers of nematodes were found in the central portion of nodules where they apparently were feeding and reproducing on bacteroids. Noninfected nodules were compact and whitish to light pink, whereas infected nodules were enlarged, brown, and often had obvious holes. Although this nematode had no consistent detrimental effect on growth or numbers of nodules that developed during short-term experiments, N_2 fixation generally was depressed by 80 to 90% compared to plants with noninfected nodules.

Additional key words: bacteria, histology.

Several workers have been concerned with the role of microbivorous nematodes in plant diseases. Steiner in 1953 (16) suggested that nematodes such as *Rhabditis* spp. and *Panagrolaimus* spp. are not "wholly nonpathogenic and may be carriers of bacteria and fungal spores." Jensen (8) showed that fungal spores ingested by four microbivorous species remained viable upon defecation. Chantanao and Jensen (4) found that plant pathogenic bacteria survived passage through nematode intestines. These reports provide evidence that the phytopathogenic microorganisms may be carried in a nematode's digestive system and be introduced into plant tissues during feeding. Pepper (14) described a case in which high populations of a *Panagrolaimus* sp. were found associated with *Helminthosporium vagans* in leaves of turfgrass. This melting-out disease was more severe where the nematodes occurred, but no causal agent was identified.

Paramonov (10) described two ecological groups of microbivorus (saprophagous) nematodes, eusaprobes and dyssaprobes. Eusaprobic nematodes feed on decaying organic matter and bacterial flora in saprobic foci of plant tissues. These may be involved, as Steiner (16) suggested, in dissemination of phytopathogenic microorganisms. The other ecological group described by Paramonov (10), dyssaprobic nematodes, includes *Panagrolaimus* spp. and *Cephalobus* spp. These have evolved more toward parasitism of plants and may enter tissues by means of mechanical destruction. They feed on saprobic media or the bacterial flora that become established subsequent to penetration.

Nematode-microorganism interactions on plants are not limited to pathogenic bacteria and fungi. Parviz et al. (11) demonstrated the ability of *Pristionchus lheritieri* to introduce *Rhizobium japonicum* into roots of soybean. Thus the nematode may be able to enhance nodulation in legumes.

In experiments involving interactions of *Rhizobia* with plant parasitic nematodes, we observed high numbers of unidentified microbivorous nematodes in nodules of Wando pea inoculated only with a *Rhizobium* sp. Preliminary studies were begun with *Rhabditis* sp., *Panagrolaimus* sp., *Acrobeloides buetschlii,* and *Pristionchus lheritieri*. Since *Acrobeloides buetschlii* was found in much higher numbers than the other species in roots of pea, detailed studies were initiated on the interaction of *A. buetschlii* with *Rhizobium leguminosarum* on Wando pea.

MATERIALS AND METHODS

Five-day-old pea seedlings, *Pisum sativum* L., were transplanted singly from vermiculite to 15-cm diameter clay pots containing a mixture of 670- and 246-μm (35- and 65-mesh) sand (1:1, v/v) (15). At the time of transplanting, 200 mg of a commercial preparation of *Rhizobium leguminosarum* (Frank) Frank (Nitragin®, The Nitragin Co., Milwaukee, Wisc.) were added around the base of the hypocotyl. Plants were grown in a temperature-controlled greenhouse, and watered with one-half strength Hoagland's nutrient solution (6) minus N, or tap water as needed. Supplementary N ($NaNO_3$) was added during the first 2 weeks after transplanting. One week after transplanting, seedlings were inoculated with water suspensions of *Acrobeloides buetschlii* (deMan) Steiner and Muhrer collected in Baermann

funnels, from quarter-strength nutrient-agar cultures of nematodes grown on a mixture of bacteria. Check plants (*Rhizobium* alone) were included in each experiment.

Data recorded at the termination of each experiment included: top weight; root weight; number of nodules; weight of dry nodules; mean nodule size (mg/nodule); N_2-fixing capacity per plant; nodular efficiency (N_2-fixing capacity per gram of nodule); and final nematode populations in soil, roots, and nodules. Nematode populations in the soil were extracted by an elutriation and sieving method (1). Nematode numbers in roots and nodules were determined by collecting all nematodes that emerged from tissues during 1 week in a mist chamber.

An acetylene-ethylene assay was used to determine N_2-fixing capacity (2, 5). Intact roots were removed from soil, rinsed briefly in water, and placed in plastic bags (0.102 × 127.0 × 152.4 mm) which were sealed with a modified Sears Model 259 bag sealer (Sears, Roebuck and Co., Chicago, Ill.). The heat bars of the sealer were covered with mylar to prevent bags from sticking, and the voltage was reduced to 50 volts with a rheostat. Bags were then filled with a gas mixture containing approximately 0.04% CO_2, 18% O_2, 72% Ar, and 10% C_2H_2 and incubated 30 minutes when a subsample was taken for analysis, using an F and M Model 700 gas chromatograph. Temperatures used were: oven, 50 C; detector, 250 C; injection port, 140 C. The flow of the helium carrier gas was 30 cm^3 per minute through a 1.5-mm × 178-cm column, containing Porapak N® (Waters Assoc., Inc., Framingham, Mass.) (7). Representative retention times for ethylene and acetylene were 1.5 and 2.5 minutes, respectively. Peak heights were converted (on the basis of a standard curve) to μmoles of ethylene per plant per hour.

For histopathology, roots and nodules were removed and fixed in FAA (formalin-ethanol-acetic acid) (9), dehydrated in a *t*-butyl alcohol series, and embedded in Tissueprep® (Fisher Scientific Co., Fair Lawn, N. J.), sectioned (12-18 μm) with a rotary microtome, and stained with Triarch Quadruple Stain (George H. Conant, Triarch Incorporated, Ripon, Wisc.). Stains incorporated into tissues were safranin (1% in 50% ethanol), crystal violet (1% aqueous), orange G (0.4% in clove oil), and fast green (1% in absolute ethanol).

All experiments were set up in a completely randomized design and data were subjected to analysis of variance. Least significant difference values were computed where warranted.

RESULTS

In the first experiment, nematode inoculum consisted of 20,000 adults and larvae of *A. buetschlii* per pot. Flowers were removed from all plants to maintain vegetative growth throughout the experiment. Three replicates were harvested 6 and 8 weeks after transplanting, and five replicates were harvested 10 weeks after transplanting. Nitrogen-fixing capacity for 8 and 10 weeks was 0.8 μmoles ethylene produced per plant per hour for inoculated plants as compared to 7.3 and 12.6 μmoles for noninoculated plants, respectively. No differences were observed between the N_2-fixing capacities of inoculated and noninoculated plants at the 6-week harvest. The nematode had no effect on growth or numbers of nodules at any harvest. Populations of nematodes in roots did not change between the 6- and 10-week harvests, averaging 500 per root system, whereas populations in nodules increased from 188 to 971 nematodes per plant between the 6- and 10-week harvests. The maximum final population of nematodes in soil was 6,800 per pot.

In a subsequent experiment using 8,000 adults and larvae per pot and only one harvest, five *Rhizobium* check plants and four nematode-*Rhizobium* inoculated plants were harvested 8 weeks after transplanting. Flowers were removed from all plants during the experiment. *Acrobeloides buetschlii* inhibited N_2-fixing capacity and nodulation and depressed nodular efficiency (Experiment II, Table 1). Shoot growth was enhanced by nematode infection compared to checks.

The third experiment was designed to evaluate effects of removing the flowers from plants on the nematode-plant interaction. Single plants were inoculated with 10,000 adults and larvae. All flowers were removed from one-half of the plants. Four replicates of each treatment were harvested at 8 and 10 weeks after transplanting, and four additional plants were harvested to determine the nematode populations in individual nodules. From 30 to 40 nodules were removed at random from each plant and placed in water with ethoxyethyl mercury chloride (Aretan®) (100 μg/ml) for 24 hours. Emerging adults and larvae were counted.

This nematode was found to inhibit N_2 fixation, but the relative inhibition declined with increasing age of plants except where flowers were removed (Experiment III, Table 1). At the 8-week harvest, *A. buetschlii* inhibited N_2-fixing capacity and depressed nodular efficiency. At 10 weeks, only plants without pods showed this inhibition, but there were significant differences in nodular weight, size, and N_2-fixing capacity between plants with pods and plants without pods. Nodule weights, N_2-fixing capacity, and nodular efficiency were lower for plants with pods than for plants without pods.

Populations of nematodes in the soil, roots, and nodules did not change between the two harvests and the two treatments of Experiment III. The overall means were 30,000 per pot in soil, 1,700 per root, and 940 in nodules per plant.

Populations of *A. buetschlii* extracted from single nodules of Wando pea varied considerably as evidenced by the high coefficient of variation (169-194%) and a range of 0 to 788 nematodes. The means were 63 and 35 nematodes per nodule for the 8- and 10-week harvests, respectively. Mean numbers of nematodes per milligram dry nodule weight were 30 and 27 on 8- and 10-week-old plants, respectively.

The histological investigations confirmed invasion of roots and nodules of pea by *A. buetschlii*. High numbers of nematodes were found in the central portion of nodules, and disruption of the bacteroid-filled cells by *A. buetschlii* was evident where healthy nodules were compared with infected nodules [Fig. 1-(A-B)]. Serial sections through infected nodules revealed cavities that developed after nodules had been invaded by *A. buetschlii* [Fig. 1-(C-D)]. Nematodes were found in the cortex of roots, but never in the vascular tissue (Fig. 1-E).

Marked morphological changes were observed in nodules from 10-week-old plants [Fig. 1-(F-G)].

TABLE 1. Effects of the microbivorous nematode, *Acrobeloides buetschlii,* on growth, nodulation, and N_2-fixing capacity of Wando pea inoculated with *Rhizobium leguminosarum*

Treatment	Dry weights: Tops (g)	Dry weights: Roots (g)	Dry weights: Nodules (mg)	Nodules (no./plant)	Nodule size (mg/nodule)	N_2 fixation: Capacity[a]	N_2 fixation: Efficiency[b]
Experiment II:							
(8-week harvest)							
Rhizobium	1.5	0.3	154	99	1.6	11.5	72
Rhizobium + nemas	2.0	0.4*[c]	105	71*	1.5	1.9**[c]	21*
Experiment III:							
(8-week harvest)							
With pods:							
Rhizobium	3.4	0.4	160	69	2.3	8.7	60
Rhizobium + nemas	3.6	0.6	206	86	2.6	0.3**	2**
Without pods:							
Rhizobium	3.2	0.7	237	76	3.1	7.8	33
Rhizobium + nemas	3.6	0.7	244	102	2.6	1.2*	5
(10-week harvest)							
With pods:							
Rhizobium	5.6	0.6	236†[d]	113	2.1††	1.5††	6††
Rhizobium + nemas	5.7	0.6	97*†††[d]	64	1.5††	0.3	3
Without pods:							
Rhizobium	5.5	1.0	392	90	4.8	9.3	24
Rhizobium + nemas	5.8	1.5	434	106	4.2	1.8**	4**

[a]Micromoles of ethylene produced per plant per hour.
[b]Micromoles of ethylene produced per gram nodule per hour.
[c]Asterisks, * and **, indicate that nematode-inoculated treatments are different from *Rhizobium* checks at $P = 0.05$ and $P = 0.01$, respectively.
[d]Daggers, † and ††, indicate that plants with pods are different from plants without pods within a particular harvest and treatment at $P = 0.05$ and $P = 0.01$, respectively.

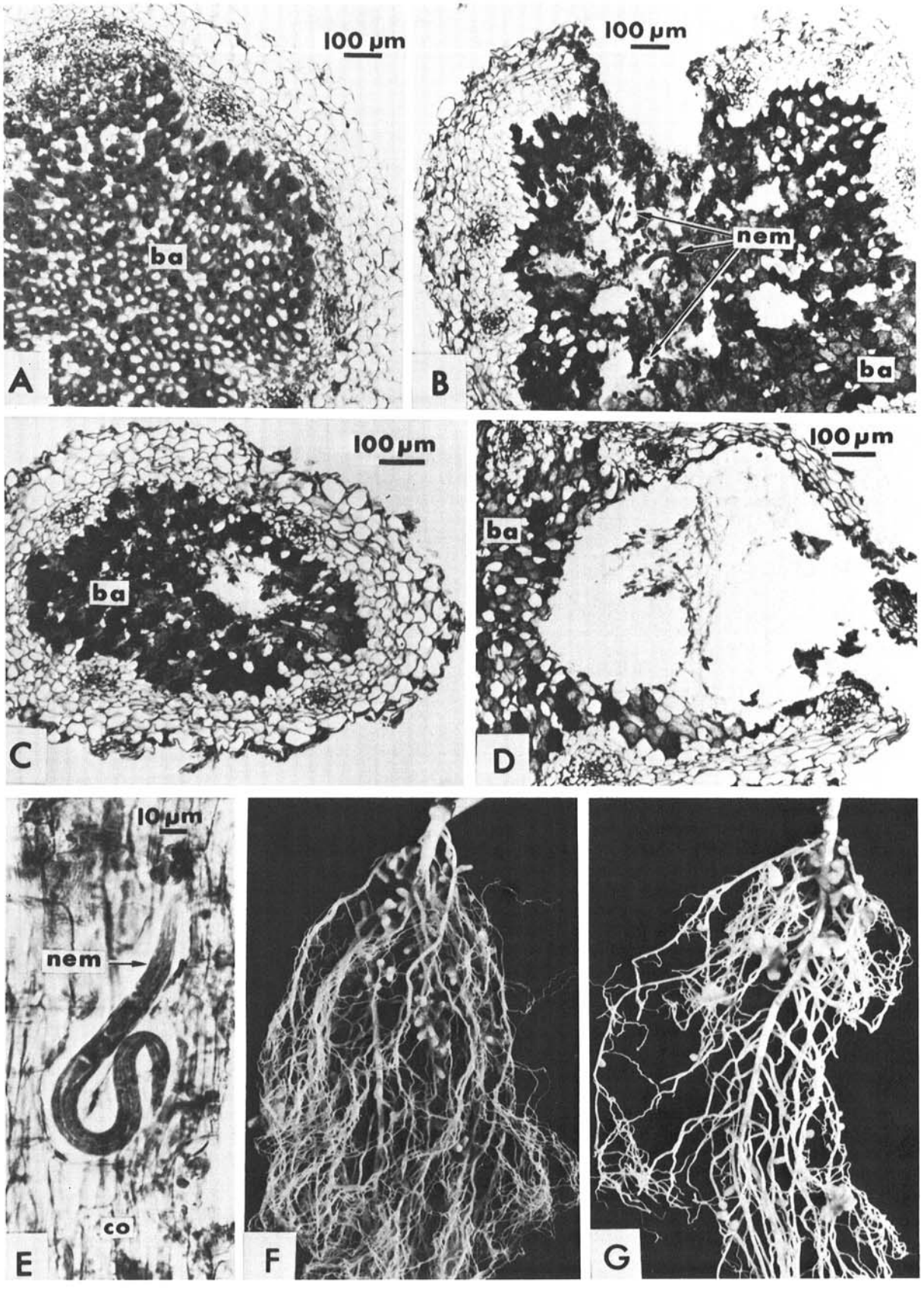
Noninfected nodules appeared compact and had a whitish to light pink color, whereas infected nodules were swollen and brown. Instead of having the white tips characteristic of healthy nodules, tips of infected nodules often appeared to have disintegrated. Though not evident in Fig. 1-(F-G), some nodules had obvious cavities in their sides as a result of nematode entry.

DISCUSSION

Previous studies of nematode-plant interactions generally have been confined to stylet-bearing plant-parasitic nematodes. The possible importance of nonstylet-bearing nematodes in crop production has been studied little and is usually related to indirect effects such as those mentioned in the introductory section of this paper. The ability of *A. buetschlii* to enter and reproduce in nodules is evident from the high numbers of all stages of nematodes found in nodules. This nonstyleted nematode is associated with the bacteroid-filled cells in nodules, and usually is not found in the cortex of the nodule. Although invasion of root cortex occurred, large numbers of *A. buetschlii* were not observed as in nodules. The association of *A. buetschlii* with the bacteroids suggests that *Rhizobium* is the food source for the nematode. Preliminary studies showed that *A. buetschlii* reproduces readily on *R. leguminosarum* in monoxenic culture, but definitive in vivo studies were not undertaken. If the nematodes feed on the *Rhizobium* in nodules, this would represent a special case of a dyssaprobe as described by Paramonov (10).

Although N_2-fixing capacity was consistently lower for nematode-infected plants compared with *Rhizobium* checks, plant growth was not affected. This may have been due to the short period the nematode had to act on the plant, or it may mean that the nitrogen available in pea seeds and the nitrogen added during the first 2 weeks of growth, along with the amount that was fixed before the nodules became infected by nematodes was sufficient to sustain the plant. Pate (12, 13) showed that soon after pods were set the N_2-fixing capacity in pea nodules declined. This was found to be true in our experiments and would further limit the damage that could have been caused by *A. buetschlii* by shortening its effective period.

Fig. 1-(A to G). Morphological and anatomical changes caused by invasion of the microbivorous nematode, *Acrobeloides buetschlii,* into roots and nodules of Wando pea inoculated with *Rhizobium leguminosarum*. **A)** Section through healthy nodule. **B)** Section through nodule containing nematodes. **C-D)** Serial sections through an infected nodule showing a cavity that developed after invasion by *A. buetschlii*. **E)** Tangential section through cortex of root containing a nematode. **F)** Noninoculated *Rhizobium*-check root. **G)** Nematode-inoculated root. Legend: ba = bacteroids; co = cortex; nem = nematode.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The traditional concept of parasitism of plants by nematodes may be inappropriate for nodules of legumes. Microbivorous nematodes such as *A. buetschlii* may be better adapted to feed and reproduce in these symbiotic structures than many stylet-bearing endoparasites. Such organisms possibly should be called facultative parasitic nematodes since they can destroy or disrupt the activities of these very important symbionts of legumes.

Studies involving perennial legumes such as alfalfa and clover should prove interesting because these plants have nodules morphologically similar to those in pea. Long-term experiments in the greenhouse and field may reveal significant damage of the rhizobia by microbivorous nematodes, which may prove to be one of the causes of the decline of these crops in the southeastern USA (3).

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