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# INTERACTIONS BETWEEN *MELOIDOGYNE INCOGNITA* AND ENDOPHYTIC BACTERIA IN COTTON AND CUCUMBER

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**Summary**—Greenhouse studies with cotton and cucumber were made to determine the effects of inoculation of the parasitic nematode *Meloidogyne incognita* on population dynamics of indigenous bacterial endophytes and introduced endophytic bacterial strains JM22 (*Enterobacter asburiae*) and 89B-61 (*Pseudomonas fluorescens*) applied as seed treatments. Internal communities of endophytic bacteria in roots were generally largest in the presence of *M. incognita*. Recovery of JM22 from cucumber roots was positively, but not significantly, associated with soilborne nematode inoculum size, exeept at 2 weeks after inoculation. The internal populations of 89B-61 applied to seed also increased with nematode applications. The diversity of indigenous bacterial endophytes changed within 7 d after *M. incognita* inoculation. Species richness and diversity of endophytic bacteria were slightly, but not significantly, greater for nematode-infested plants than for non-infested plants. *Alcaligenes piechaudii* and *Burkholderia pickettii* occurred only in nematode-infested plants, whereas *Brevundimonas vesicularis* was mainly isolated from nematode-free plants. *Agrobacterium radiobacter* and *Pseudomonas* spp. were the most common taxa found in both treatments, accounting for a total of 41% and 37% of the community for non-inoculated and inoculated plants, respectively. JM22 colonized cotton roots internally and was also found in high numbers on the root surface around nematode penetration sites and on root galls where the root tissue had been disruptured due to gall enlargement. Single cells of JM22 were attached to the cuticle of *M. incognita* juveniles. Endophytic bacteria and *M. incognita* form complex associations, and an understanding of these associations will aid efforts to develop and manage microbial communities of endophytic bacteria for practical use as biocontrol agents against plant-parasitic nematodes and soilborne pests and pathogens. 

## INTRODUCTION

Sedentary plant-parasitic nematodes and pathogenic bacteria form complex associations within the host plant (Sitaramaiah and Pathak, 1993). Nematodes can play an important role in predisposing the host plant to invasion by secondary pathogens. This is particularly true for wounds produced by nematodes which favor bacterial colonization of the root surface and their introduction into the root tissue (Zutra and Orion, 1982; Bookbinder *et al.*, 1982). Biochemical changes within the host tissue associated with nematode infestation presumably result in an environment beneficial for pathogenic bacteria (Sitaramaiah and Pathak, 1993). Plant-parasitic nematodes can negate bacterial wilt resistanee (Reddy *et al.*, 1979) and act as vectors by carrying external bacterial cells into the plant tissue (Crosse and Pitcher, 1952). Enhanced disease severity has often been observed when plants were infected with nematodes several days or weeks prior to exposure to bacterial pathogens, compared to simultaneous infection of plants with both organisms (Johnson and Powell, 1969; Sitaramaiah and Sinha, 1984).

Some studies have indicated antagonistic relationships between plant-parasitic nematodes and bacterial pathogens. Stewart and Schindler (1956) found that *Ditylenchus* spp. suppressed carnation wilt caused by *Pseudomonas caryophylli*. Lucas and Krusberg (1956) noticed reduced symptom development caused by *Xanthomonas solanacearum* on tobacco in the presence of *Tylenchorhynchus claytoni* and concluded that in this particnlar case, the ectoparasitic nematode was unable to penetrate deep into the xylem tissues, and that symptom expression might be different for sedentary nematodes such as *Meloidogyne incognita*.

Pathogenic baeteria form only a fraction of the total microbial community within plant tissue. In general, a broad spectrum of predominantly Gram-negative bacteria is reported for several crops including alfalfa (Gagné *et al.*, 1987), cotton (Misaghi and Donndelinger, 1990), potato (De Boer and Copeman, 1974), and tomato (van Peer *et al.*,

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1990). These saprophytic bacteria coexist with the host plant without doing any apparent harm to the plant. Saprophytic bacteria with apparently no deleterious effect on plant growth are also referred to as endophytes. Indigenous endophytic communities cover a broad spectrum of bacterial species as shown by Melnroy and Kloepper (1995), who isolated a total of 47 different species from cotton plants throughout a growing season.

Overall, little is known about the interactions of plant-parasitic nematodes and saprophytic endophytes. Based on the results observed for pathogenie bacteria, larger endophytic communities in the presence of plant-parasitic nematodes would be expected. Considering the alterations in plant physiology due to nematode infection, alterations in the endophytic microbial community may be expected. Our objectives were to investigate the effect of nematode numbers on total endophytic bacterial communities, evaluate the significance of *M. incognita* as a vector for the model endophyte *Enterobacter asburiae*, strain JM22, and study the effect of *M. incognita* on indigenous endophytic baeteria and fungi in cotton and cucumber. Results from these studies should contribute to a better understanding of the complex assoeiation among sedentary plant-parasitic nematodes, saprophytic endophytes and the host plant. This information should eventually lead to the development of new biocontrol approaches against plant-parasitic nematodes based on antagonistic endophytes.

## MATERIALS AND METHODS

### *Bacterial inoculum*

*Enterobacter asburiae* strain JM22 was originally isolated from cotton (Musson *et al.*, 1995). This strain was chosen because of its colonization potential of various crops including cucumber, cotton and bean (Mahaffee *et al.*, 1994; Quadt-Hallmann and Kloepper, 1996). *Pseudomonas fluorescens* strain 89B-61 originated from canola (*Brassica napus*) and was provided by Agrium, Inc. (Saskatoon, Canada). 89B-61 had been shown to reducc disease incidence caused by *Pseudomonas syringae* pv. *lachrymans* on cucumber (Wei *et al.*, 1996) and of *M. incognita* on cotton and cucumber (Hallmann *et al.*, 1997). Bacteria were stored at −80°C in tryptic soy broth (TSB) (Difco, Detroit, MI) with 20% glycerol and plated on tryptic soy agar (TSA) (Difco, Detroit, MI) before use. For seed treatment, approximately 100 mg f.w. bacteria from a culture grown on TSA for 24 h at 27°C were suspended in 2 ml of a 2% aqueous methyl cellulose solution. The seeds were exposed to this suspension for 30 min and then dried under a laminar flow hood. Final bacterial numbers were approximately log 9 cfu seed$^{-1}$ for both cotton and cucumber. For application of the bacteria as a soil drench, bacterial cells from 10 TSA plates completely covered with bacterial colonies were harvested and suspended in 6.0 l sterilc 10mM potassium phosphate buffer, pH = 7.0 (PB). Fifty ml aliquots of this log 8.8 cfu bacterial suspension ml$^{-1}$ were poured into the soil around each plant.

### *Nematode inoculum*

A *Meloidogyne incognita* strain highly aggressive on cotton was provided by Jim Starr (College Station, TX, U.S.A.) and was maintained on "Rowden" cotton (*Gossypium hirsutum*) and tomato (*Lycopersicon esculentum* cv. "Rutgers"). Inoculum was produced by extracting eggs from galled cotton roots using the NaOCl technique of Hussey and Barker (1973). The egg suspension was agitated in tap water to stimulate hatching. For experiments using eggs as inoculum, the egg suspension was passed within 24 h of extraction over a sieve combination of 45 µm and 25 µm mesh size. Eggs collected on the 25 µm sieve were essentially free of remaining plant debris and were used immediately. Second-stage juveniles were extracted after 10 d agitation by a modified Baerman technique (Rodríguez-Kábana and Pope, 1981). Eggs or juveniles were inoculated by pipetting 1 ml tap water containing the requisite amount of inoculum into the root zone around the host plant.

### *Effects of Meloidogyne incognita on introduced endophytes*

Three experiments were made to test the hypothesis that nematode infections of roots would enhance internal colonization of endophytes. Two experiments used JM22 as a model systemic endophyte and one experiment used 89B-61 as a model root cortical endophyte. For experiment 1 cucumber (*Cucumis sativus* cv. "SMR58") seeds treated with JM22 were planted into pasteurized sand at 3 seeds pot$^{-1}$. After emergence the seedlings were thinned to 1 pot$^{-1}$ and fertilized with 20 ml Peter's solution (20-20-20, Scotts-Sierra, Maryvilles, OH, U.S.A.). Freshly-hatehed juveniles of *M. incognita* were inoculated 8 d after planting. The experiment consisted of six inoculum sizes (0, 300, 700, 1000, 1500 or 3000 *M. incognita* juveniles plant$^{-1}$) and two sampling times (13 or 26 d after *M. incognita* inoculation). Each treatment was replicated eight times, and the pots were arranged as a randomized complete block. The experiment was terminated 34 d after planting. Shoot fresh weight and root fresh weight were reeorded, and the gall index was rated on a scale from 0–10, where 0 = no infestation and 10 = maximum nematode infestation (Zeck, 1971). The roots were then surface-disinfected in 1.05% NaOCl (Clorox® diluted 1:5 with tap water) for 60 s followed by three washes in sterile PB. Roots were then impressed on TSA as a sterility check for bacteria on the surface, and tritu-

rated five times in (w/v) sterile PB with mortar and pestle. Following serial dilution, 100 µl of the macerate was plated on 5% TSA, and the plates were incubated for 24 h at 27°C. For strain confirmation 10 randomly-chosen bacterial colonies were tested by ELISA using JM22-specific antibodies (Quadt-Hallmann and Kloepper, 1996). Colonies resembling the ones identified as JM22 were counted to determine cfu $g^{-1}$ root tissue.

Experiment 2 was performed with "Rowden" cotton seed treated with JM22 and inoculated with *M. incognita* eggs 8 d after planting. The experiment consisted of five treatments (0, 600, 2500, 8000 or 15 000 *M. incognita* eggs $plant^{-1}$) and four sampling times (d 5, 10, 17 or 27 after nematode inoculation). Each experimental unit was replicated 5 times and the experiment was set up as a randomized complete block. At each sampling time shoot fresh weight, root fresh weight and endophytic numbers of JM22 (log cfu $g^{-1}$ fresh wt root) were recorded. Additionally, the number of galls were counted for each sampling time except for the first sampling. Confirmation of an internal population of JM22 was done as described before.

Experiment 3 used the potential biocontrol agent *Pseudomonas fluorescens* strain 89B-61 applied as a seed treatment on cotton cv. "Rowden" in a randomized complete-block design. Eleven days after planting, the seedlings received an additional soil drench of 50 ml bacterial solution (log 8.8 cfu $ml^{-1}$), and *M. incognita* was inoculated 17 d after planting at a rate of 12 000 eggs $plant^{-1}$. The experiment consisted of three treatments (control, 89B-61 or 89B-61 + *M. incognita*) and two sampling times (11 or 27 d after nematode inoculation), each replicated eight times. Twenty-seven days after nematode inoculation fresh shoot weight, fresh root weight, number of galls and endophytic population of 89B-61 (log cfu $g^{-1}$ fresh wt root) were recorded. Internal colonization of 89B-61 was confirmed by ELISA using polyclonal antibodies (Quadt-Hallmann *et al.*, 1997).

### *Effects of Meloidogyne incognita on indigenous endophytes of cotton*

To test the hypothesis that plant-parasitic nematodes enhance community size and diversity of indigenous endophytes within plants, an experiment was conducted with field soil from the E.V. Smith Research Center, Horticulture Unit of the Alabama Agricultural Experiment Station near Shorter, Alabama. The soil had been fumigated with methyl bromide 20 months before experimental use. The soil was a sandy loam with pH = 6.1 and organic matter content < 1.0% (w/w). The soil was mixed with siliceous sand 1:1 (w/w), hereafter referred to as soil, and potted in 1000 $cm^3$ cylindrical pots. Six subsamples of 100 $cm^3$ of the final substrate used in the experiment confirmed the absence of plant-parasitic nematodes. Mean numbers of saprophytic nematodes were 104 individuals 100 $cm^{-3}$ substrate. Cotton cv. "Rowden" was planted with three seeds $pot^{-1}$ and thinned to 1 plant $pot^{-1}$ after emergence. The plants were fertilized once with 25 ml Peter's solution (20:20:20). The experiment consisted of two treatments, a non-inoculated control and inoculation with *M. incognita*. Each treatment was replicated 10 times. Two plants which did not pass the sterility check following surface disinfestation were discarded and a total of eight replications were finally evaluated. Each seedling was inoculated with 3000 freshly hatched juveniles of *M. incognita* 21 d after planting. The experiment was terminated 30 d after planting. Fresh weights of shoot, root and of the first two leaves were separately recorded. In addition, numbers of galls and gall index were evaluated. The roots and first 2 leaves were surface disinfected as described before, and the macerate was plated on 0.05 strength TSA and Ohio-agar (Johnson and Curl, 1972) for bacterial and fungal recovery, respectively. The plates were incubated at 27°C for 48 h (bacteria) or 72 h (fungi).

To describe indigenous bacterial diversity single colonies were isolated from the 0.05 strength TSA plates. Triangular segments were randomly drawn at the bottom of each plate containing approximately 40 colonies. Starting from the center and moving towards the periphery of the plate, each colouy within the segment was transferred onto TSA. The isolates were tested for purity and were stored at −80°C in TSB + 20% glycerol. Twenty-five isolates from the first six replications were identified based on fatty acid methyl-ester analysis (Sasser, 1990; McInroy and Kloepper, 1995). The bacterial community was characterized using four diversity indices: the number of species present (species richness, N0), the abundance of species within each sample (evenness, E5), and two combinations of richness and evenness defined as Hill's diversity numbers N1 and N2, with N2 more than N1 representing very abundant species (Ludwig and Reynolds, 1988). Based on our experiment with a total of 25 identified strains per sample, richness could range from 1 (all strains are identified the same) to 25 (all strains are different species). Similar, Hill's diversity numbers N1 and N2 can range from 1 (one strain dominant) to 25 (all strains are equally abundant) with N2 < richness. The evenness index (E5) is equal to 1 when all species are equally represented and approaches 0 when a single species is dominant.

### *Meloidogyne incognita–endophyte association*

To determine if nematodes could distribute endophytes, 1 ml with 2000 freshly-hatched second-stage juveniles of *M. incognita* was added to 1 ml bacterial suspension of JM22 (log 8.5 cfu $ml^{-1}$). After exposure for 15 min 100 µl aliquots containing ap-

proximately 10 juveniles were pipetted onto a total of 15 plates of 0.05 strength TSA. The plates were kept uncovered under the laminar flow hood until excess water of the bacterial-nematode suspension was evaporated. Non-treated nematode juveniles were used as control for initial bacterial contamination. The plates were then covered and incubated up to 96 h at 27°C. The plates were observed daily under a dissecting microscope for juvenile movement across the plate and bacterial growth along the nematode tracks.

A secoud approach was based on the adhesive potential of bacterial spores on the juvenile cuticle. Five-thousand *M. incognita* juveniles were kept in a suspension of JM22 (log 8.5 cfu $ml^{-1}$) for 30 min; juveniles kept in sterile PB served as a control. The nematode-bacteria suspension was poured over a 20 µm sieve, and excess bacterial solution was gently washed off with tap water for 30 s. The juveniles were pipetted into cylindrical microsporous capsules (Electron Microscopy Sciences, Fort Washington, PA, U.S.A.) of 12.5 mm × 12.5 mm, which were set into a 20 ml glass beaker. Protected within these capsules, the juveniles were prepared for scanning electron microscopy (SEM). The porous material of the capsules allowed good infiltration with the various chemical solutions. Nevertheless, to avoid excessive juvenile losses due to escape or retention within the porous material, it was necessary to coat the cavity with microscopic lens paper. The juveniles were fixed with Karnovsky solution for 2 h at room temperature (Karnovsky, 1965). The nematodes were then washed six times (15 min each) with sodium cacodylate buffer (200 mM, pH 7.2), post-fixed with 2% osmium tetroxide for 2 h at room temperature, and again washed six times. Dehydration was conducted with an increasing ethanol-to-dd$H_2O$ series and with eritical point drying using $CO_2$. After complete drying, the juveniles were mounted on metal stubs covered with sticking tape and were sputter-coated with gold. Thereafter, they were evaluated for bacterial presence with a digital SEM (DSM 940, Zeiss, Germany).

Studies concerning the *M. incognita*–endophyte association on the rhizoplane and within the root tissue were made using SEM and transmission electron microscopy (TEM). "Rowden" cotton seed was treated with JM22 and planted into steam-sterilized sand. After 8 d the seedlings received an additional soil drench with JM22 (log 8.8 cfu $ml^{-1}$), followed by inoculation with 5000 juveniles of *M. incognita* 24 h later. At 15 and 21 d after planting, root tips and side roots were embedded for microscopic evaluation as described above for SEM. Samples for TEM were prepared without a postfixation of osmium tetroxide to receive a better preservation of antigenic properties. After dehydration with ethanol, root tissues were embedded in LR-White acrylic resin. Polymerization was carried out in gelatin capsules for 48 h at 50°C. Inmunogold labeling of JM22 in ultrathin sections followed the procedure described by Quadt-Hallmann and Kloepper (1996) using specific antibodies against this bacterium.

*Statistical analysis*

Data on bacterial colonization were transformed into $\log_{10}$ cfu $unit^{-1}$ (g fresh wt, ml or seed) to make treatment variances homogenous. Data were analyzed according to standard procedures including SAS general linear model and least significant difference (LSD) using SAS software (SAS Institute, Cary, NC). Unless otherwise stated, all differences referred to in the text were significant at the 5% or lower level of probability.

## RESULTS

*Effect of Meloidogyne incognita on introduced endophytes*

Recovery of JM22 from cucumber roots varied depending on time and nematode inoculum density (Fig. 1). For experiment 1 maximum bacterial recovery was reached with 1000 juveniles (log 5.53 cfu $g^{-1}$ fresh wt root) for the first sampling time 13 d after inocnlation, and at 3000 juveniles (log 5.5 cfu $g^{-1}$ fresh wt root) for the second sampling tiine. In general, bacterial recovery was higher at the second sampling time except for 1000 juveniles. Absence of *M. incognita* resulted in bacterial recovery of log 2.96 and log 3.89 cfu $g^{-1}$ fresh wt root at 13 and 26 d after nematode inoculation, respectively. The applied concentrations of *M. incognita* eggs or juveniles did not affect shoot and root growth of cucumber, but the gall index increased significantly with increasing nematode inoculum density (Table 1).

In experiinent 2, recovery of JM22 from cotton roots was maximum with nematode inocula of 2000 (d 5), 8000 (d 10), 2000 (d 17) or 15 000 eggs (d 27) and averaged log 3.39, 3.55, 4.09, and 4.05 cfu $g^{-1}$ fresh wt root, respectively (Fig. 2). Based on the sampling tiine, bacterial recovery was highest 17 d after nematode inoculation, regardless of the inoculum density except for 15 000 eggs, when the last sampling time yielded the highest bacterial recovery. Bacterial colonies on TSA were uniform for experiments 1 and 2 and all randomly-selected colonies were confirmed as JM22. Increasing nematode inoculum size did not affect plant growth (data not shown); however, number of galls increased significantly with increasing nematode inoculum size (Fig. 3).

In experiment 3, inoculation with *M. incognita* increased recovery of 89B-61 from cotton roots signifieantly (log 4.59 cfu $g^{-1}$ fresh wt root) compared to application of 89B-61 alone (log 3.31 cfu $g^{-1}$)

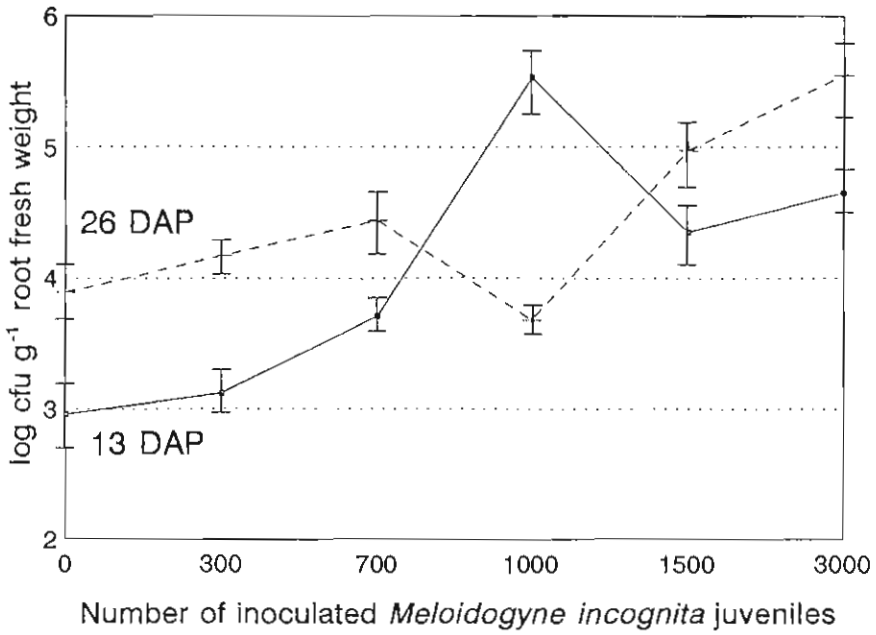


Fig. 1. Effect of inoculum numbers of *Meloidogyne incognita* juveniles on numbers of JM22 within the roots of cucumber at two sampling times 13 d and 26 d after planting (DAP). Data are means and standard errors from eight replicate plants each.

(Table 2). The lowest bacterial populations were detected for untreated plants with log 1.10 cfu $g^{-1}$ fresh wt root. For plants inoculated with 89B-61, cross contamination with unknown bacterial strains was less than 1% as confirmed by ELISA. Application of 89B-61 significantly increased shoot weight of the cotton seedlings, but had no effect on root growth (Table 2). Plants inoculated with *M. incognita* averaged 110 galls per root system. However, few galls were also detected on 1 out of 8 plants in both non-inoculated treatments.

*Effects of Meloidogyne incognita on indigenous endophytes of cotton*

The community size of indigenous root endophytes was significantly greater in association with *M. incognita*, averaging log 5.81 cfu $g^{-1}$ fresh wt root compared to log 5.01 cfu $g^{-1}$ fresh wt root for non-inoculated plants (Table 3). Mean endophytic fungal communities of non-inoculated and inoculated plants were log 1.05 and log 1.46 cfu $g^{-1}$ fresh wt root, but differences were not significant. Compared with the root tissue, fewer bacterial endophytes were recovered from shoot tissue. Endophytic bacterial communities averaged log 2.79 cfu $g^{-1}$ fresh wt shoot for non-inoculated plants and log 3.09 cfu $g^{-1}$ fresh wt shoot for *M. incognita*-inoculated plants. No fungal colonies were detected within the shoot tissue of either treatment. At the end of the experiment no *M. incognita* juveniles were detected within the soil of the non-inoculated treatment, but 161 juveniles 100 $cm^{-3}$ soil were recorded for *M. incognita*-inoculated soil (Table 3). Number of saprophytic nematodes was not significantly different between the two treatments (Table 3).

Inoculation of *M. incognita* did not affect growth of the cotton seedlings, although *M. incognita* infes-

Table 1. Relation between juvenile inoculum numbers of *Meloidogyne incognita* and plant growth and root gall indices† in cucumber

| | Shoot fresh weight (g) | | Root fresh weight (g) | | Gall index |
|---|---|---|---|---|---|
| Inoculated juveniles | 13 d | 26 d | 13 DAI | 26 DAI | 26 DAI |
| 0 | 1.77a | 3.73a | 1.90a | 3.19a | 0.75d |
| 300 | 1.63a | 3.86a | 1.26a | 3.35a | 1.67cd |
| 700 | 1.29a | 4.14a | 0.95a | 3.33a | 4.00bc |
| 1000 | 1.29a | 3.83a | 1.13a | 2.84a | 4.00bc |
| 1500 | 1.43a | 3.77a | 1.19a | 2.83a | 5.50ab |
| 3000 | 1.65a | 3.97a | 1.24a | 3.33a | 7.25a |
| LSD | 0.82 | 0.69 | 1.01 | 1.04 | 2.92 |

†Gall index on a scale from 0–10 with 0 = no galls and 10 = severe gall infestation (Zeck, 1971).
Means with the same letter are not significantly different at $P = 0.05$. LSD values are for comparison of means within the column above each value. Number of replicates = 8.
DAI = Days after inoculation of *M. incognita*.

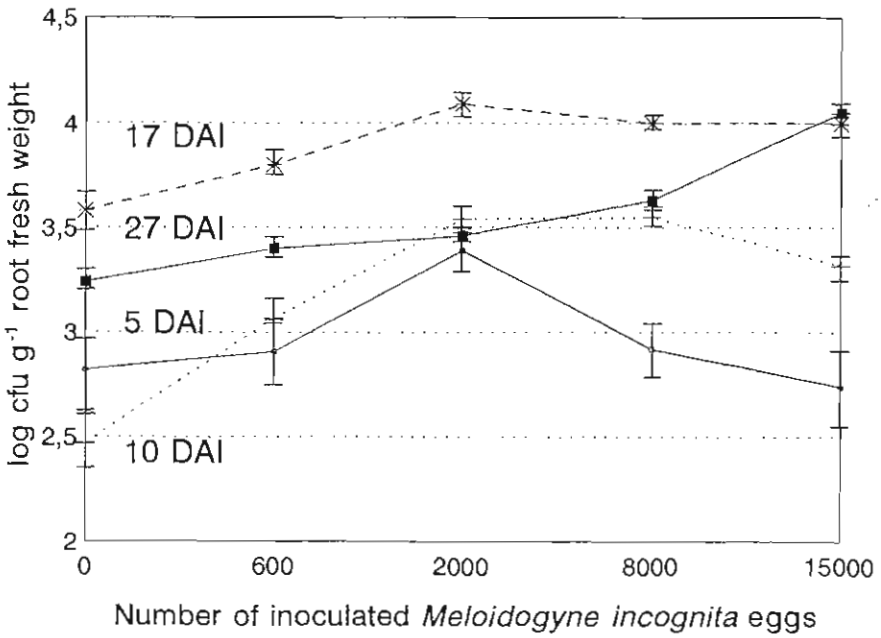


Fig. 2. Effect of inoculum numbers of *Meloidogyne incognita* eggs and sampling time on numbers of JM22 within cotton roots. Sampling times were 5, 10, 17 and 27 d after nematode inoculation (DAI). Data are means and standard errors from five replicate plants each.

tation caused a gall index of 4.9 which was equivalent to 183 galls per root system (Table 4). The shoot-to-root ratio was slightly higher for non-inoculated plants, but differences were not significant.

Table 5 gives the number of replicates positive for bacterial occurrence, mean population sizes of indigenous species and the range of bacterial frequency within one treatment. No single species dominated within any treatment. Bacterial frequency of one species ranged from 1 to 11 isolates out of 25 isolates characterized per treatment, and the total number of species recovered from one root system ranged from 9 to 14 species (data not

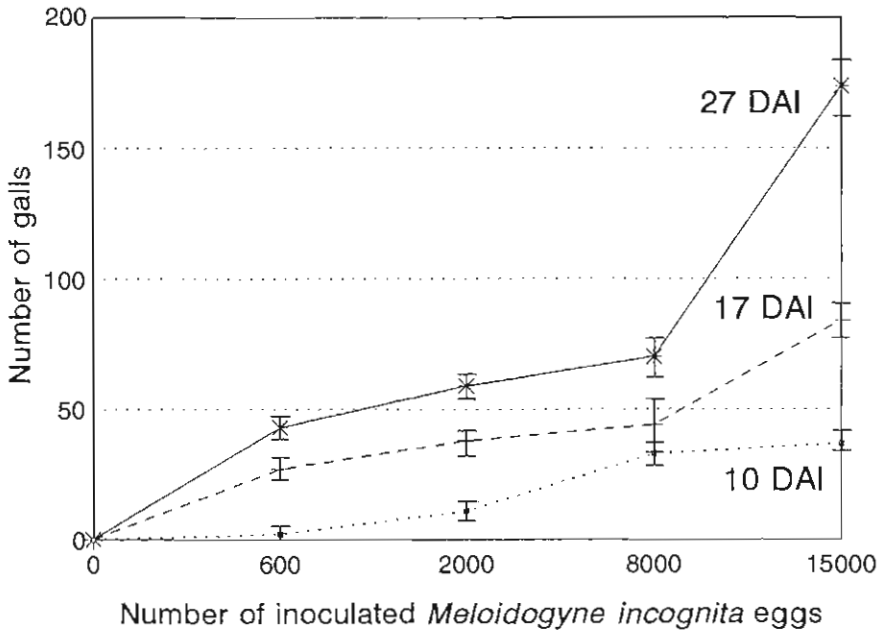


Fig. 3. Effect of sampling time and inoculum numbers of *Meloidogyne incognita* eggs on number of galls on cotton roots at 10, 17 and 27 d after nematode inoculation (DAI). Data are means and standard errors from five replicate plants each.

Table 2. Effect of *Meloidogyne incognita* on growth of cotton plants and root colonization by *Pseudomonas fluorescens* strain 89B-61

| | Shoot fresh weight (g) | Root fresh weight (g) | Number of galls | log cfu $g^{-1}$ fresh wt root |
|---|---|---|---|---|
| Control | 1.70c | 1.66a | 0.38b | 1.10c |
| 89B-61 | 2.07b | 1.35a | 0.50b | 3.31b |
| 89B-61/*M. inc.* | 2.42a | 1.59a | 110a | 4.59a |
| LSD | 0.28 | 0.51 | 22 | 1.268 |

Means followed by the same letter are not significantly different at $P = 0.05$. LSD values are for comparison of means within the column above each value. Number of replicates = 8.

shown). Gram-positive bacterial species accounted for 2.1% of the total community isolated from non-inoculated plants. No Gram-positive bacteria were recovered from *M. incognita*-infested roots. For the following comparison of bacterial community structure, the relative population size of each species is given in parenthesis. *Agrobacterium radiobacter* was most consistently isolated from both non-inoculated (21.9%) and inoculated (18.6%) plants. *Brevundimonas vesicularis* was predominant in non-inoculated plants (12.6%) compared to inoculated plants (4.0%). *Burkholderia pickettii* (8.6%) and *Alcaligenes xylosoxydans* (4.7%) were exclusively isolated from plants inoculated with *M. incognita*. *Variovorax paradoxus* was much more common in inoculated (11.2%) than in non-inoculated (3.3%) plants. Overall, *Pseudomonas* spp. were commonly isolated from both treatments (18.7% non-inoculated, 18.8% inoculated plants), but this genus was represented by 8 species with none of them being dominant. The diversity indices as determined by either species richness (N0), species evenness (E5), or a combination of these factors (N1, N2) are shown in Table 6. In general, endophytic bacteria recovered from cotton roots inoculated with *M. incognita* indicated a higher diversity based on richness and diversity; however, the differences were not significant. The evenness index showed no difference between the two treatments.

### *Meloidogyne incognita–endophyte association*

A chain of bacterial colonies of JM22 was observed on the agar surface along the path of the nematode (Fig. 4). Bacterial growth occurred as far as 10 cm from the inoculation site, indicating transport of the bacteria by *M. incognita*.

The described preparation of *M. incognita* juveniles for SEM resulted in excellent structural preservation of the nematode tissue. Single cells of JM22 were found on the cuticle of *M. incognita* juveniles (Fig. 5). However, the low numbers of bacterial cells attached to the juveniles surface seem to exclude any specific binding mechanism. Nevertheless, *M. incognita* juveniles treated with sterile PB did not have any bacterial cells on their cuticle.

Large numbers of JM22 were observed on the surface of nematode galls, especially where the root epidermis had disrupted due to gall expansion (Figs 6 and 7). Comparable areas analyzed by TEM also showed an accumulation of bacterial cells around necrotic plant cells in the vicinity of galls (Fig. 8). Observations at higher magnification of bacteria within these areas demonstrated an intensive gold label of the bacterial membranes confirming the bacteria as JM22 (Fig. 9). No bacteria were detected in association with the anterior end of the nematode within the plant tissue or inside giant cells.

## DISCUSSION

In general, internal numbers of indigenous and applied endophytes were higher in *M. incognita*-infested cucumber and cotton than in non-infested plants. A similar association is known for plant-parasitic nematodes and pathogenic bacteria, resulting in additive plant damage (Khan, 1993). Total numbers of internal JM22 and 89B-61 were up to log 4 cfu $g^{-1}$ root which is similar to populations reported for other endophytes (Misaghi and Donndelinger, 1990; Bell *et al.*,

Table 3. Effect of *Meloidogyne incognita* on numbers of indigenous endophytic bacteria and fungi isolated from cotton roots and shoots, and nematode numbers in the soil

| | Microbial numbers (log cfu $g^{-1}$ fresh wt) | | | | Nematode numbers in soil | |
|---|---|---|---|---|---|---|
| | Root | | Shoot | | | |
| | Bacteria | Fungi | Bacteria | Fungi | *M. incognita* | Saprophytes |
| Control | 5.01b | 1.05a | 2.79a | ND† | 0b | 148a |
| *M. incognita* | 5.81a | 1.46a | 3.09a | ND | 161a | 155a |
| LSD | 0.60 | 0.73 | 0.89 | | 48 | 54 |

†ND = non-detectable, numbers were below detection limit of 50 cfu $g^{-1}$.
Means followed by the same letter are not significantly different at $P = 0.05$. LSD values are for comparison of means within the column above each value. Number of replicates = 10.

Table 4. Effect of *Meloidogyne incognita* on plant growth, shoot-to-root ratio and number of galls on "Rowden" cotton

| | Shoot fresh weight (g) | Root fresh weight (g) | Gall index | Number of galls | Shoot-to-root ratio |
|---|---|---|---|---|---|
| Control | 6.36a | 4.27a | 0b | 0b | 1.61a |
| *M. incognita* | 6.35a | 4.36a | 4.9a | 183a | 1.48a |
| LSD | 1.59 | 1.27 | 0.53 | 20 | 0.48 |

Means followed by the same letter are not significantly different at $P = 0.05$. LSD values are for comparison of means within the column above each value. Number of replicates = 10.

1995; Quadt-Hallmann and Kloepper, 1996). However, this number is much lower than for populations of pathogenic bacteria which can range from log 7 cfu $g^{-1}$ fresh weight, e.g. for *Clavibacter michiganensis* subsp. *sepedonicus* on tomato (Tsiantos and Stevens, 1986), to log 9–10 cfu $g^{-1}$ fresh weight under severe disease pressure as reported for *Pseudomonas solanacearum* on tomato, eggplant or aubergine (Grimault and Prior, 1994).

Table 5. Indigenous bacterial endophytes in cotton roots as affected by *Meloidogyne incognita*

| | Control | | | +*M. incognita* | | |
|---|---|---|---|---|---|---|
| Bacterial species | Replicates* positive | Mean† | Range‡ | Replicates* positive | Mean† | Range‡ |
| Gram-positive | | | | | | |
| *Micrococcus halobius* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *M. luteus* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *Pediococcus pentosaceus* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| Subtotal | | 2.1 | | | 0 | |
| Gram-negative | | | | | | |
| *Acetobacter pasteurianus* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *Acidovorax delafieldii* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *Aeromonas salmonicida* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *Agrobacterium radiobacter* | 5 | 21.9 | 0–11 | 4 | 18.6 | 0–10 |
| *A. rubi* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *Alcaligenes piechaudii* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *A. xylosoxydans* | 0 | 0 | 0 | 3 | 4.7 | 0–3 |
| *Azospirillum brasiliense* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *Brevundimonas diminuta* | 3 | 3.3 | 0–2 | 0 | 0 | 0 |
| *B. vesicularis* | 6 | 12.6 | 1–6 | 3 | 4.0 | 0–3 |
| *Burkholderia cepacia* | 3 | 2.7 | 0–2 | 4 | 5.3 | 0–4 |
| *B. pickettii* | 0 | 0 | 0 | 5 | 8.6 | 0–5 |
| *Gluconobacter oxidans* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *Chryseobacterium balustinum* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *Comamonas acidovorans* | 1 | 0.7 | 0–1 | 4 | 4.0 | 0–2 |
| *C. testosteroni* | 1 | 1.3 | 0–2 | 2 | 1.3 | 0–1 |
| *Cytophaga johnsonae* | 2 | 1.3 | 0–1 | 2 | 2.0 | 0–2 |
| *Erwinia carotovorum* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *E. chrysanthemi* | 1 | 0.7 | 0–1 | 0 | 0 | 0 |
| *Flavobacterium resinovorum* | 4 | 4.7 | 0–3 | 4 | 7.9 | 0–5 |
| *Kluyvera cryocrescens* | 2 | 1.4 | 0–1 | 0 | 0 | 0 |
| *Neisseria mucosa* | 1 | 1.3 | 0–2 | 0 | 0 | 0 |
| *Pantoea stewartii* | 1 | 1.3 | 0–2 | 2 | 2.0 | 0–2 |
| *Photobacterium angustum* | 1 | 2.7 | 0–4 | 0 | 0 | 0 |
| *Phyllobacterium myrsinaceorum* | 2 | 1.3 | 0–1 | 2 | 2.0 | 0–2 |
| *P. rubiacearum* | 3 | 8.6 | 0–8 | 3 | 4.0 | 0–3 |
| *Pseudomonas aeruginosa* | 4 | 7.3 | 0–4 | 1 | 2.7 | 0–4 |
| *P. fluorescens* | 1 | 3.3 | 0–5 | 4 | 4.0 | 0–2 |
| *P. marginalis* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *P. mendocina* | 0 | 0 | 0 | 2 | 2.7 | 0–2 |
| *P. putida* | 4 | 4.7 | 0–3 | 5 | 6.0 | 0–4 |
| *P. rubrisubalbicans* | 3 | 2.0 | 0–1 | 2 | 1.3 | 0–1 |
| *P. stutzeri* | 1 | 0.7 | 0–1 | 1 | 0.7 | 0–1 |
| *P. syringae* | 1 | 0.7 | 0–1 | 1 | 0.7 | 0–1 |
| **Pseudomonas, total** | 6 | 18.7 | 1–11 | 6 | 18.8 | 3–9 |
| *Sphingomonas capsulata* | 2 | 2.7 | 0–3 | 0 | 0 | 0 |
| *S. paucimobilis* | 2 | 1.3 | 0–1 | 0 | 0 | 0 |
| *Stenotrophomas maltophilia* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *Variovorax paradoxus* | 3 | 3.3 | 0–3 | 5 | 11.2 | 0–7 |
| *Vibrio cholerae* | 2 | 2.7 | 0–3 | 0 | 0 | 0 |
| *Xanthobacter agilis* | 0 | 0 | 0 | 1 | 0.7 | 0–1 |
| *Xanthomonas campestris* | 1 | 0.7 | 0–1 | 1 | 0.7 | 0–1 |
| Subtotal | 97.9 | | | | 100 | |
| Number of species | 33 | | | | 29 | |

*Number of replicates with positive occurrence of bacterial species (out of six).
†Mean number of bacteria based on total population (= 100%).
‡Range of bacterial frequency within the six replications (total of 25 isolates per replication).
Bacterial spectrum is based on the characterization of 25 isolates per replication and 6 replications per treatment.

Table 6. Species diversity indices for indigenous bacterial endophytes of cotton as affected by *Meloidogyne incognita*

| | Richness N0* | Diversity N1† | Diversity N2‡ | Evenness E5§ |
|---|---|---|---|---|
| Control | 8.0 | 8.3 | 6.4 | 0.71 |
| *M. incognita* | 9.2 | 9.1 | 7.1 | 0.74 |
| LSD¶ | 3.07 | 2.28 | 3.27 | 0.24 |

*Richness is a measure for number of species.
†N1 is a measure for abundant taxa.
‡N2 is a measure for very abundant taxa.
§E5 approaches 1 when all species are equally represented in a sample.
¶LSD values are for comparison of means within the column above each value. The differences were not significant at $P = 0.05$.

Sampling time is another important factor in community studies of endophytic bacteria since bacterial communities are usually larger at 3–4 weeks than 1–2 weeks after nematode inoculation. The amount of nematode inoculum and sampling time also interact with each other. For plants analyzed less than 2 weeks after nematode inoculation highest bacterial numbers were reached at median rates of nematode inoculum, whereas when analyzed 3–4 weeks after nematode inoculation highest bacterial numbers were reached at maximum nematode inoculum levels. This might indicate changes in plant physiology and nutrient availability with establishment of nematode feeding sites and nematode development (Bird, 1975). However, our knowledge concerning total amount of nutrients available for endophytic bacteria as well as the composition and exact localization of these nutrients within the plant tissue is still very limited and needs further investigation.

Increased numbers of JM22 following *M. incognita* inoculation were restricted to the root tissue, but were not observed in aerial plant parts where there had been no plant cell disruption. The nematode probably creates entrance avenues for JM22 but might also carry single bacterial cells over short distances directly into the root tissue. Assimilates translocated basipetally due to nematode infestation (Bird, 1975) might act as an additional food source for JM22 and therefore explain the increased numbers of JM22.

The effect of *M. incognita* on indigenous bacteria in cotton was determined 7 d after nematode inoculation. Although several hundred bacterial isolates were identified, the diversity indices did not show any significant difference between the treatments. As the distribution of endophytic bacteria is often inconsistent and of high variability between different plants, formulating general conclusions becomes difficult. However, *Agrobacterium radiobacter* and

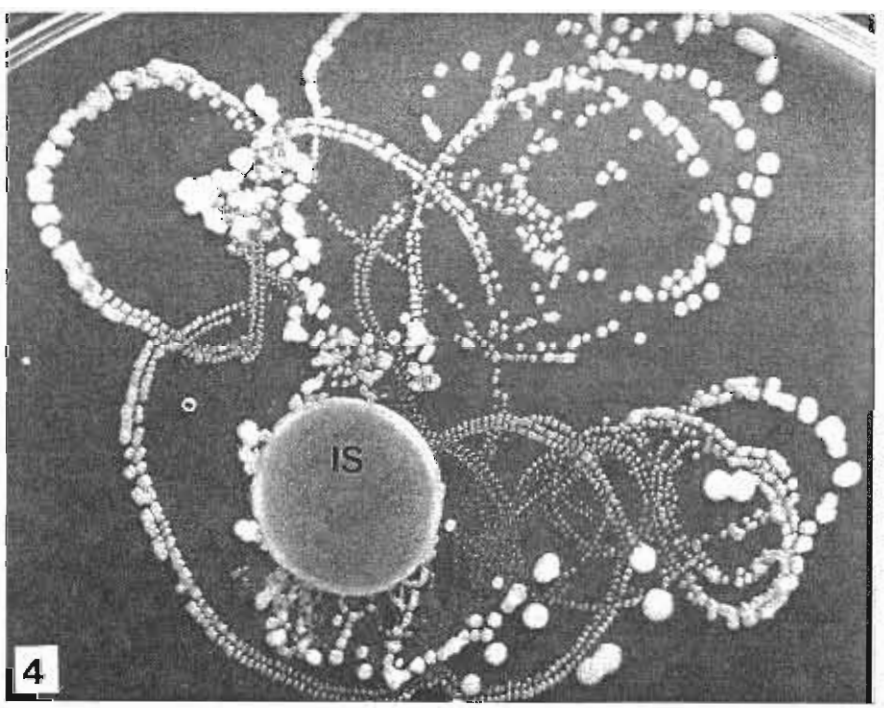


Fig. 4. Growth of JM22 along the tracks of *Meloidogyne incognita* on water agar as bacterial cells were carried by the juveniles from the inoculation spot (IS) in the center of the agar plate towards the periphery.

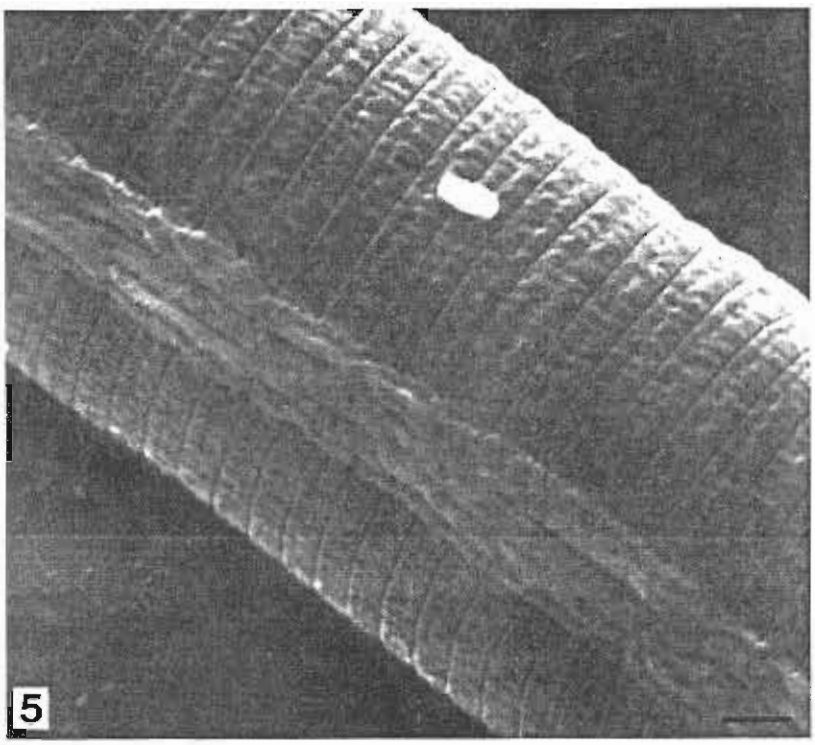


Fig. 5. Scanning electron microscopy of JM22 attached to the cuticle of a *Meloidogyne incognita* juvenile after several washing steps. Scale bar = 2 µm.

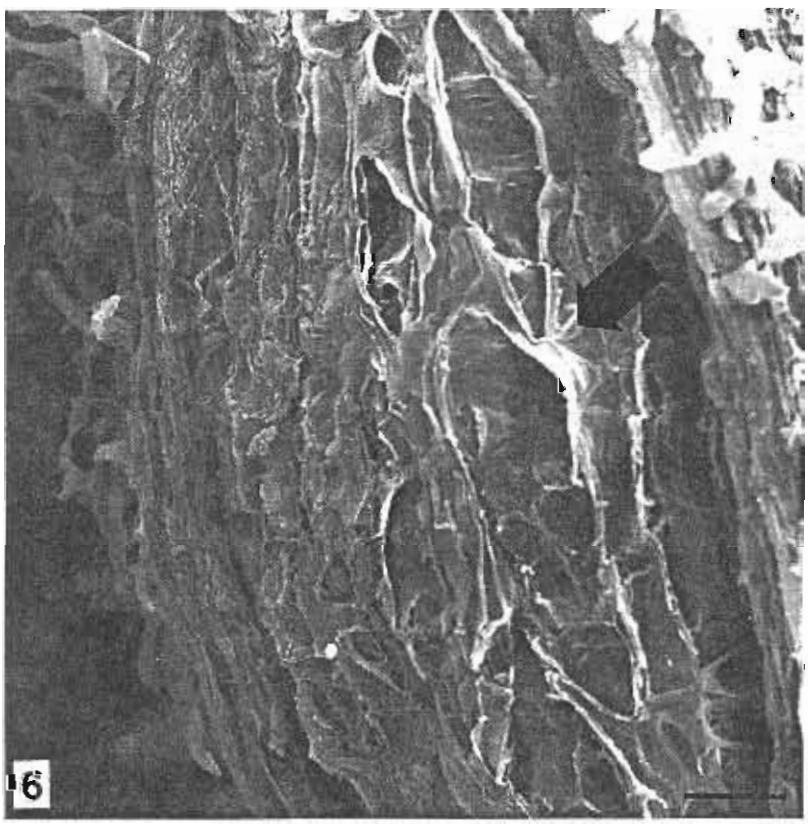


Fig. 6. Scanning electron microscopy of a disrupted root epidermis of cotton during gall formation. Bacteria, apparently JM22, are located on the surface of the newly-formed root gall. Arrow indicates area of higher magnification shown in Fig. 7. Scale bar = 100 µm.

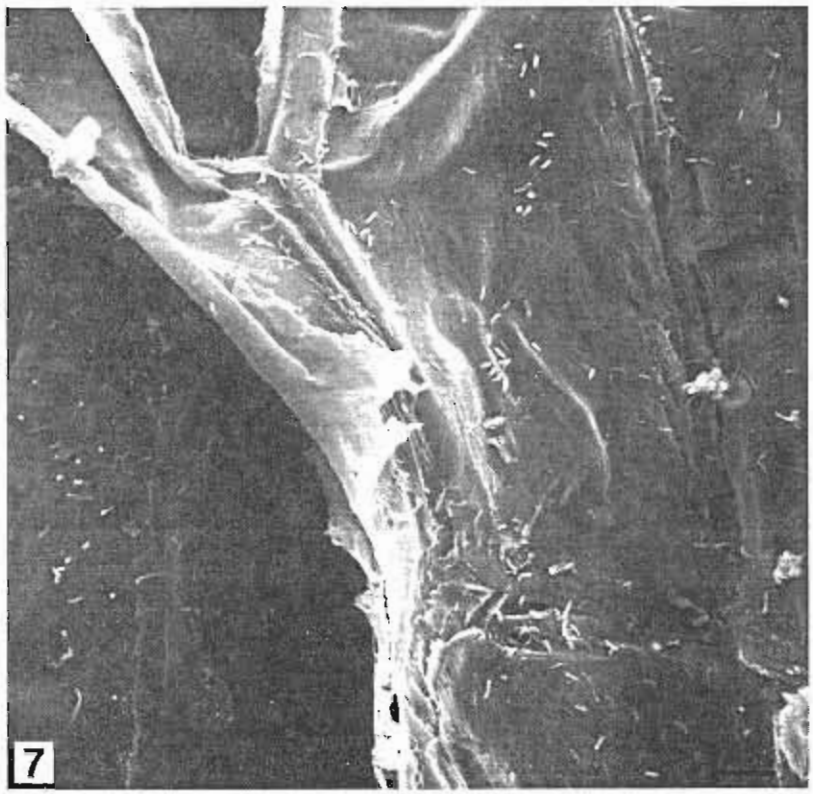


Fig. 7. Higher magnification of Fig. 6 showing JM22 located on the surface of the newly-formed root gall. Scale bar = 10 μm.

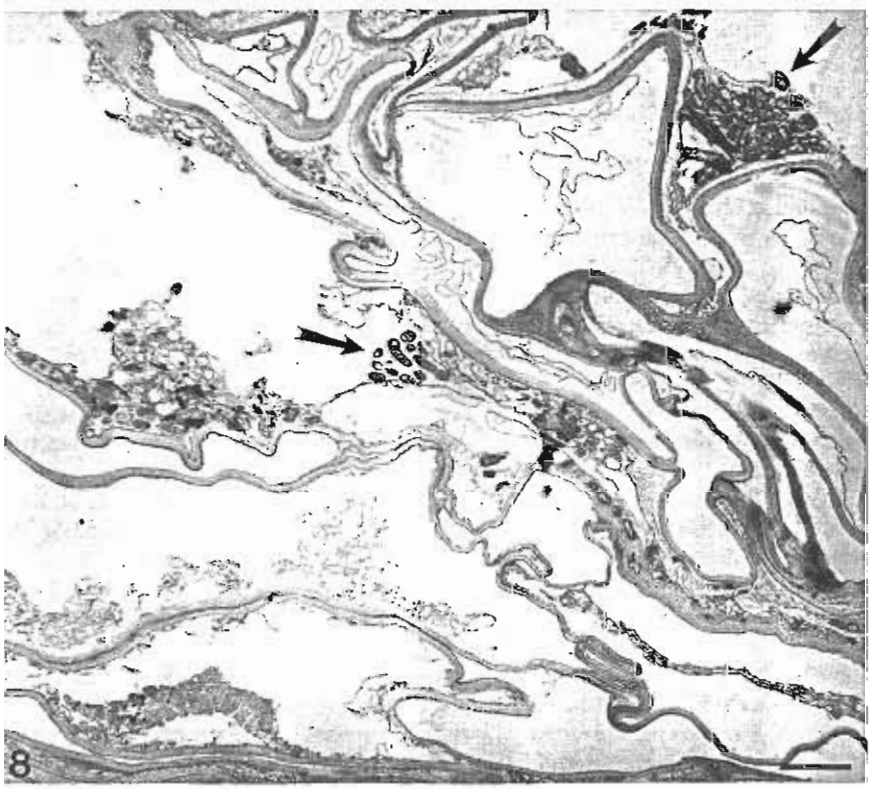


Fig. 8. Transmission electron microscopy of ultrathin sections of damaged cotton root tissue probably due to penetration by M. incognita. Bacterial cells of JM22 are located on the root surface and around necrotic cells in the root cortex (arrows). Scale bar = 10 μm.

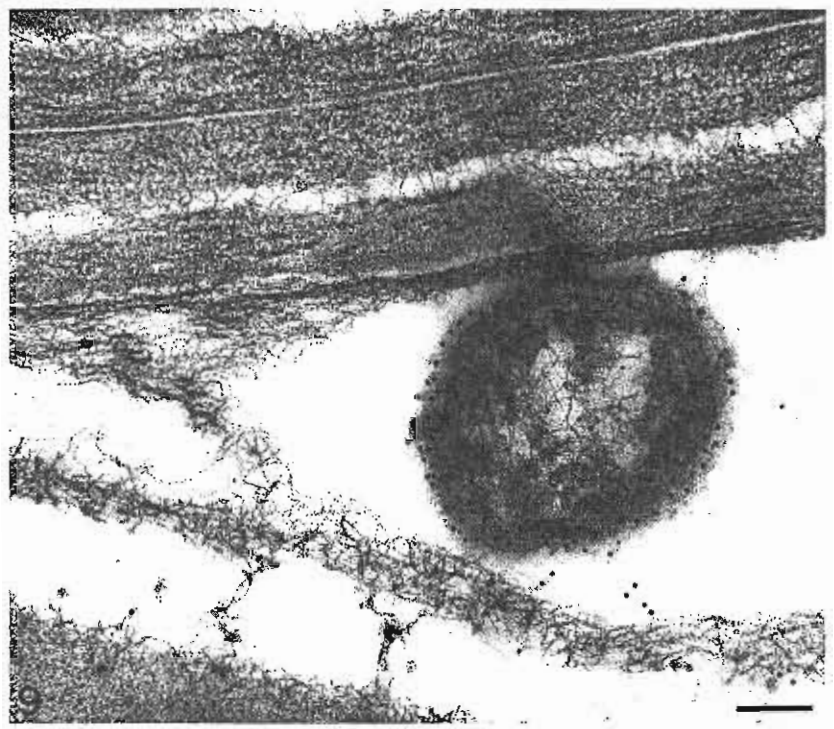

Fig. 9. Transmission electron microscopy of ultrathin sections of a cotton root inoculated with JM22 showing JM22 in the root cortex. Intensive gold label of the bacterial membrane of JM22 confirms strain identity (application of primary antibodies at a dilution of 1:500 and secondary antibodies coupled with colloidal gold at 1:40). Scale bar = 0.5 µm.

*Pseudomonas* spp. were dominant in both treatments. *Alcaligenes xylosoxydans* and *Burkholderia pickettii* were only isolated from *M. incognita*-infested roots. Assuming that endophytic bacterial species vary in their specific nutritional requirements, the presence or absence of single species might represent alterations in plant physiology due to nematode infestation, but also might differ with nematode species. A novel biocontrol strategy would be to search for microorganisms that are antagonistic to nematodes, but which will be favored in roots infected by *M. incognita*. These microorganisms would be competitive colonizers following nematode infection, but would survive in low numbers when the nematode became absent. After antagonistic communities have become established within the root tissue they would have regular adverse effect on nematode development.

Single cells of JM22 remained adhering to the cuticle of *M. incognita* after several washing steps. However, considering the large bacterial inoculum in the starting solution the final number of attached bacteria was low, indicating a weak and non-specific attachment. Nevertheless, a few bacteria carried by *M. incognita* into root tissue might be sufficient to support bacterial colonization. Tests have shown that systemic colonization and growth of JM22 up to log 4 cfu $g^{-1}$ root followed use of small amounts of inoculum of log 5 cfu $seed^{-1}$ (Hallmann, unpublished data). However, we do not know if the total number of bacteria colonizing the internal root tissue following seed application at these low rates is even close to the number of bacteria carried by juveniles into the root tissue.

Passive transport of bacteria by *M. incognita* was demonstrated for JM22 on agar thus confirming observations by Kalinenko (1936) for pathogenic bacteria. It is assumed that the water film around the juvenile favours this unspecific bacterial transport. Since there may be similar conditions in the field, plant-parasitic nematodes could serve as vectors for saprophytic bacteria.

Besides growth of JM22 within roots due to *M. incognita* infection, high numbers of the saprophytic endophytes were also found externally around disrupted root tissue, such as the penetration sites of nematode juveniles and galled tissue. Immunogold labeling confirmed these bacteria as JM22. It is assumed that plant cell disruption could lead to leakage of nutrients favorable for these bacteria.

Our results indicate that the total number of bacteria as well as the species composition of the endophytic communities change due to *M. incognita* infection. However, it is still unknown how these changes in microbial community structure affect the plant–nematode association in terms of nutrient availability, space competition or antagonistic activity towards the nematode. A better understanding of these microbial shifts will enable development of novel strategies for the biocontrol

of plant-parasitic nematodes based on the introduction of antagonistic endophytes. The fact that the plant may serve as a protective niche for the endophytic bacterium emphasizes endophytes as ideal biocontrol agents for plant-parasitie nematodes as has already been demonstrated for insect control in aerial plant parts (Tomasino *et al.*, 1995).

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