MANAGEMENT OF *MELOIDOGYNE INCOGNITA* IN COTTON, USING STRAINS OF THE BACTERIUM *GLUCONACETOBACTER DIAZOTROPHICUS*

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Summary. Seven strains of the bacterium *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) were assessed for their antagonistic efficacy against root-knot nematode (*Meloidogyne incognita)* infecting cotton *in vitro* and *in planta*. Cell free culture supernatant of the seven strains of *G. diazotrophicus* reduced egg hatching by more than 95% and exhibited a paralyzing effect on the infective juveniles of *M. incognita* within 24 hours. All the strains were found to excrete ammonia and volatile fatty acid during growth. Greenhouse screening of the bacterial strains indicated that all except strain 35-45 significantly enhanced the growth of nematode-infected cotton plants. These strains also reduced nematode multiplication to various extents, ranging from 36 to 69%. Evaluation of the two most effective strains in the field for three years revealed that strain 35-47 persistently and significantly increased cotton yield and reduced root-knot index. Strain Co 99-70, although found to be effective in the greenhouse, failed to produce responses in the field.

Cotton (*Gossypium* spp.) is susceptible to root-knot nematode (*Meloidogyne* spp.), which affect yields (Sasser and Freckman, 1987). During the last decade, various rhizobacteria have been evaluated for their antagonistic effect on a variety of plant-parasitic nematodes, including *Meloidogyne incognita* (Kofoid *et* White) Chitw. (Kloepper *et al*., 1992). Antibiosis effects, causing decreased nematode infestation in the soil eco-system, have been observed as a consequence of a range of microbial metabolites produced during bacterial growth. Among the volatile metabolites, ammonium ions have been shown to repel *M. incognita* juveniles (Castro *et al*., 1990) and acetic acid inhibited egg hatching (Bansal and Bajaj, 2003) and the movement of infective juveniles (Djian *et al*., 1991).

Since an acid-tolerant, endophytic rhizobacterium, *Gluconacetobacter diazotrophicus* Yamada, Hoshino *et* Ishikawa is known to produce volatile fatty acid and fix atmospheric nitrogen (Cavalcante and Dobereiner, 1988), the biocontrol potential of this species against the root-knot nematode, *M. incognita*, infecting cotton (*G. hirsutum* L.) was evaluated *in vitro*, in a wire netted greenhouse and in nematode infested fields.

MATERIALS AND METHODS

The seven strains of *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus* Gillis, Kerters, Hoste, Kroppenstedt, Stephan, Teineria, Dobereiner *et* Deley), viz. 35-45, 35-47, 767-31, 767-33, 767-50, 767-57 and Co 99-70, used in the present study were obtained from the culture collection, Department of Microbiology, CCS HAU, Hisar. The cultures were maintained on LGI (lacto-glucose infusion) medium (Cavalcante and Dobereiner, 1988) slants. Cell-free culture supernatants of the strains were assayed for their effect on egg hatching and motility of *M. incognita* juveniles. For this, each strain was grown in LGI broth for 4 days at 30 °C on a rotary shaker and centrifuged at 10,600 g for 20 min. The cell-free supernatants obtained were stored at 5 °C and used as and when required. Batches of three freshly picked egg masses were placed on a small piece of nylon mesh and kept in solid watch glasses, to which five ml of culture supernatant were added, and incubated at 28 °C. Emerging juveniles were counted daily for 10 days. Percentage hatch was calculated with reference to the average number of eggs per egg mass of the respective treatment. Fresh supernatant was added after each observation. Distilled water and LGI medium were used as controls for comparison. Each treatment was replicated three times. To study juvenile motility, a suspension of 500 freshly hatched juveniles ml^{-1} of sterile water was prepared. About 50 juveniles in 0.1 ml of water and one ml of culture supernatant were added to each well of a tissue culture plate. Water and LGI medium were included as controls and three replicates were used per treatment. Plates were maintained at 28 °C. Each well was examined microscopically after 24 h and the juveniles that appeared paralyzed and did not respond to a touch with a needle were considered "immobilized" and counted. The amount of ammonia excreted by different strains of *G. diazotrophicus* during four days of growth was estimated directly in the culture supernatant using the method of Narula *et al.* (1981). Volatile fatty acid produced by each bacterial strain was estimated by steam distillation (Jain *et al*., 1981) of the cell-free supernatants and titration of each distillate

Table I. Chemical composition of cell-free culture supernatant of various strains of *Gluconacetobacter diazotrophicus* and their effect on *Meloidogyne incognita* egg hatching and juvenile mobility.

Strain	pH	NH_4^+ (µg ml^{-1})	Volatile acidity* (ppm)	% juveniles immobilized within 24 h	% egg hatched/egg mass during 10 days
35-45	3.57	0.91	420	100 (85.91)	2.1 (8.31)
35-47	3.63	2.23	240	90 (73.62)	1.7 (7.45)
767-31	3.51	0.52	570	100 (85.91)	3.7 (11.07)
767-33	3.72	0.81	390	100 (85.91)	2.5 (9.05)
767-50	3.52	0.86	690	100 (85.91)	5.0 (12.84)
767-57	3.54	0.65	570	100 (85.91)	1.7 (7.45)
Co 99-70	3.62	2.07	180	100 (85.91)	1.3 (6.52)
Lacto-glucose infusion(LGI)	6.02	0.00	0	10 (18.38)	95.2 (77.79)
Water	7.10	0.00	0	0 (4.05)	98.0 (81.93)
CD at 5%	-	-	-	(6.64)	(3.12)

* Equivalent to acetic acid.
Figures in brackets are corresponding transformed values.

against N/40 sodium hydroxide solution. Total acidity (ppm) equivalent to acetic acid was calculated.

Surface sterilized cotton (*G. hirsutum*) cv. H-1098 seeds were immersed in a culture suspension (10^8 cells ml^{-1}) of each strain separately for 30 min and sown in earthen pots filled with 3 kg of steam sterilized sandy loam soil. Five days after emergence, the seedlings were thinned to one plant per pot and a suspension of one thousand freshly hatched juveniles of *M. incognita* in 10 ml of water was poured around the exposed roots, covered with soil and watered lightly. Each treatment was replicated five times and the pots were arranged in a completely randomized block design on benches in a greenhouse. Quarter strength sterilized Sloger's nitrogen-free salt solution was used for watering the pots. The recommended dose of fertilizers (N = 88 kg and P = 30 kg ha^{-1}) was applied. Fifty days after inoculation, plant growth parameters, numbers of root knots, egg masses per root system and eggs per egg mass were recorded.

Based on laboratory and pot studies, strains 35-47 and Co 99-70 were selected for evaluation of their antagonistic efficacy against *M. incognita* in naturally infested fields for three years, at different locations. For each treatment, five replicated field plots of 10 m × 15 m were prepared. Bacterial inocula for field trials were prepared by growing each bacterial strain in LGI broth followed by mixing them separately with steam sterilized wood charcoal powder (150 mesh) to obtain culture inocula containing 10^8 cells g^{-1} charcoal powder. Cotton cv. H-1098 seeds were treated with wood charcoal based bacterial culture of each strain separately, at the rate of 200 g per 5 kg of seeds and sown. Recommended agronomic practices were followed. Plants treated with Carbofuran 3G at the rate of 1 kg a.i. ha^{-1} and untreated controls were also maintained for comparison. At harvest, cotton yield and root-knot index (1-5 scale) were recorded.

To study the colonization behaviour of *G. diazotrophicus*, inoculated and uninoculated plants were uprooted at the end of the experiment. Rhizospheric soil, adhered soil, root-tips, epidermal layer and vascular bundles were separated. To collect rhizosperic soil (soil particles that are loosely attached to roots), roots were shaken to remove loosely attached soil particles where as adhered soil (soil particles that are embedded in the root mucigel) was brushed gently. Thereafter, roots were surface sterilized using dilute hydrogen peroxide and epidermal layer (outer epidermal layer with internal cortical tissue) was scratched with sterile dissection blade. Vasicular bundles were removed by tearing the surface sterilized roots. Each sample was transferred individually to sterilized LGI broth and incubated for 2-7 days at 30 °C. Change in colour of the culture broth from green to yellow was recorded as an indication of the presence and growth of *G. diazotrophicus*.

For statistical analysis, the percentage values of juvenile hatching and motility were subjected to angular transformation before analysis of variance (ANOVA). Results are reported as transformed and untransformed figures. The data from the greenhouse and field experiments were also subjected to analysis of variance and LSD at P = 0.05 was used to compare the means between treatments.

RESULTS

In general, exposure of *M. incognita* egg masses to the cell-free culture supernatants of different strains of *G. diazotrophicus* suppressed nematode hatch (Table I). The cumulative percent hatch ranged between 1.3 and

Table II. Effect of various strains of *G. diazotrophicus* on *M. incognita* infection and growth of cotton plants.

Treatment	Galls/plant	Egg masses/plant (A)	Eggs/egg mass (B)	Total no. of eggs/plant (AxB)	Plant height (cm)	Shoot weight (g)	Root weight (g)
Control (nematode only)	235	130	340	44200	25.0	8.3	4.2
Nematode + 35-45	175 (26.4)	98 (24.7)	288	28224 (36.2)	26.6	10.5	6.9
Nematode + 35-47	129 (45.1)	64 (50.1)	210	13440 (69.5)	35.1	14.8	9.7
Nematode + 767-31	180 (23.5)	108 (17.0)	260	28080 (36.5)	30.6	12.9	8.1
Nematode + 767-33	162 (31.1)	78 (40.0)	280	21840 (50.6)	32.4	12.6	9.0
Nematode + 767-50	160 (36.2)	90 (30.8)	280	25200 (43.0)	30.2	10.8	7.8
Nematode + 767-57	164 (30.2)	52 (60.0)	296	15392 (65.2)	34.5	13.0	8.8
Nematode + Co99-70	125 (46.8)	60 (53.9)	280	16800 (62.0)	36.6	14.9	9.2
CD at 5%	23.8	13.7	31.8	-	4.3	4.1	3.5

Data in parentheses denote % decrease relative to the control. Data are means of five replicate.

5.0 over a period of ten days of incubation compared to 98.0% and 95.2% in water and LGI medium, respectively. Furthermore, when the freshly hatched nematodes were exposed to these culture supernatants, almost all juveniles were immobilized within 24 hours, whereas all the juveniles exposed to water and 90% of those in LGI medium remained active during this period. Chemical analysis of the cell-free culture supernatants revealed a sharp lowering of pH from the 6.02 of LGI medium to around 3.5 of supernatants at the end of the fourth day of bacterial growth, indicating the acid-producing ability of the bacterium. Steam distillation of various supernatants and titration of their distillates against sodium hydroxide revealed that all the strains produced volatile fatty acid to levels ranging from 180 ppm to 690 ppm (Table I). No attempt was made to identify the fatty acids. All the strains of the bacterium were also found to excrete ammonia into the growth medium, up to concentrations of 2.23 µg ml^{-1} of supernatant.

Greenhouse screening of seven bacterial strains, indicated that, except for strain 35-45, all significantly improved growth parameters of nematode infected cotton plants (Table II). However, out of six effective strains, isolates 35-47 and Co 99-70 performed the best. In addition to plant growth improvement, bacterial inoculation also decreased root galling by up to 46.8% and reduced nematode multiplication by from 36.2% to 69.5% relative to untreated controls (Table II).

Replicated field evaluation was carried out for three years at different locations, with soils naturally infested with *M. incognita*. Of the two most effective strains of *G. diazotrophicus*, selected on the basis of results from the *in vitro* and green house studies, strain 35-47 proved the best in reducing root-knot index (RKI), from 4.2 in the control to 3.0, and increasing yield, by 38.0%, followed by Carbofuran treatment (Table III). However, strain Co 99-70 produced neither a significant increase in yield nor a decrease in RKI in the first year, so was excluded from subsequent years of the field trials. During the following two years, strain 35-47 persistently and significantly performed better, increasing yields by 47.6% and 85.4% over the uninoculated control and decreasing RKI from 4.1 and 3.6 to 2.9 and 2.3, respectively.

Preliminary studies on the colonization behaviour of *G. diazotrophicus* 35-47 on cotton plant revealed its presence on the root surface only, and not in its rhizosphere or vascular bundles.

DISCUSSION

The more pronounced nematotoxic effect of culture supernatants in comparison to the growth medium-(LGI) used in the present studies indicated the presence of antinemic metabolite(s) produced by *G. diazotrophicus* during growth. Several metabolites of microbial ori-

Table III. Effect of *G. diazotrophicus* (Gd) inoculation on cotton yield in root-knot nematode infested fields.

Crop season	Treatment	Yield (q/ha)	% yield increase over control	Root-knot index
I	Gd Co 99-70	11.5	6.5	3.9
	Gd 35-47	14.9*	38.0	3.0*
	Carbofuran	13.6*	25.9	3.2*
	Untreated (control)	10.8	-	4.2
II	Gd 35-47	15.5*	47.6	2.9*
	Carbofuran	14.9*	41.9	2.8*
	Untreated (control)	10.5	-	4.1
III	Gd 35-47	16.5*	85.4	2.3*
	Carbofuran	12.9*	44.9	3.2*
	Untreated (control)	8.9	-	3.6

* Denotes statistical significance (P = 0.05) over untreated (control) in respective crop season.

gin have been reported to possess nematotoxic properties. In addition to the paralyzing effect (Bansal *et al*., 1998) and disruption in the movement of juveniles (Djian *et al*., 1991), volatile fatty acids are known to reduce egg hatching by impairing embryogenesis (Bansal and Bajaj, 2003) of *M. incognita*. The plant growth promoting effect of this bacterium can be attributed not only to its ammonia excretion ability, evident from the results (Table I), but also to the indole acetic acid produced (Fuentes-Ramirez *et al*., 1993), which helps plants to absorb more nutrients by causing root proliferation.

These results support our previous observations (Bansal *et al*., 1998) that *G. diazotrophicus* inoculation was useful in reducing root galling due to *M. javanica* in bottle gourd. Reduced root galling in the bacterium inoculated plants is related to the volatile fatty acids produced by this bacterium, which reduced nematode egg hatch and motility to an even greater extent (Table I). Thus, fewer juveniles could invade the host plant roots, producing fewer root galls. Further, the disruption in movement of infective juveniles due to fatty acid (Djian *et al*., 1991) and diminished attractiveness of root-tips due to ammonia (Castro *et al*., 1990) excreted by the bacterium probably delayed root invasion and egg deposition, resulting in reduced egg content per egg mass, thus affecting total reproduction in comparison to the uninoculated control.

From the field evaluation study, it is evident that strain 35-47, applied as a seed treatment, persistently and significantly improved growth and yield of cotton and lowered root-knot disease intensity. The beneficial effects on plant growth produced by the bacterium appear to be mainly due to two reasons. Firstly, by directly enhancing the nutritional status of plant by providing nitrogen, and indirectly by producing root hormones (Fuentes-Ramirez *et al*., 1993) that made the plants grow faster with better developed root systems, than non-bacterized plants. Bacterial mutants lacking the ability to produce indole acetic acid failed to enhance plant growth under laboratory conditions (Dobbelaere *et al*., 1999). Moreover, rhizobacteria also help in acquiring phosphorous and potassium from soils, mainly through their effects on root morphology and physiology (Cocking, 2003). Secondly, plant growth was also enhanced due to a reduction in root-knot disease severity. This happened because the bacterium produced volatile fatty acids that suppressed nematode hatch and motility, as is evident from the *in vitro* studies, enabling the plants better to withstand nematode infestation of the soil. The reduced nematode infection, was also possibly due to interference with nematode infection sites through colonization of the root surface by the bacterium, as observed in our preliminary study. Experiments to confirm the colonization behaviour with a genetically tagged bacterium are in progress.

The failure of the strain Co 99-70 to promote yield and decrease nematode disease intensity in the field was perhaps due to its sensitivity towards soil moisture, which normally remains low in fields compared to the wet condition in the pot culture. Failure of an effective strain of *Pseudomonas* sp. in field trials was also observed by Burr *et al.* (1978). Mishustin and Naumova (1962) found that *Azotobacter* inoculation was effective in soil with moisture content more than 40% of the field capacity. In addition to soil moisture, bacterial strains apparently are also affected by soil type and the available carbon source.

It can be concluded that *G. diazotorophicus* 35-47 improved growth and yield of nematode infected cotton plants and that it can regulate root-knot nematode densities in nematode infested soils.

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Accepted for publication on 28 April 2005.