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Reproduction of *Meloidogyne javanica* on Plant Roots Genetically Transformed by *Agrobacterium rhizogenes*

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Abstract: Reproduction of *Meloidogyne javanica* was compared on several *Agrobacterium rhizogenes*-transformed root cultures under monoxenic conditions. *M. javanica* reproduced on all transformed roots tested; however, more females and eggs were obtained on potato and South Australian Early Dwarf Red tomato than on bindweed, Tropic tomato, lima bean, or carrot. Roots that grew at moderate rates into the agar and produced many secondary roots supported the highest reproduction. Numbers of females produced in cultures of transformed potato roots increased with increasing nematode inoculum levels, whether inoculum was dispersed eggs or juveniles. Females appeared smaller, produced fewer eggs, and were found in coalesced galls at the higher inoculum levels. The ratio between the final and initial population decreased sharply as the juvenile inoculum increased. The second-stage juvenile was preferred to dispersed eggs or egg masses for inoculation of tissue culture systems because quantity and viability of inoculum were easily assessed. *Meloidogyne javanica* reared on transformed root cultures were able to complete their life cycles on new transformed root cultures or greenhouse tomato plants.

Key words: Agrobacterium rhizogenes, bindweed, carrot, gnotobiotic, hairy root pathogen, inoculum level, lima bean, *Meloidogyne javanica,* monoxenic, nematode stage, potato, root-knot nematode, tissue culture, tomato.

Monoxenic culture of *Meloidogyne* spp. on root explants has been used to study resistance (7), environmental and nutritional factors affecting nematode development (5,10), feeding site histology and ultrastructure (3,18), and other aspects of host–parasite biology (6,8,15). Recently, plant roots genetically transformed by *Agrobacterium rhizogenes,* the "hairy root pathogen," were used to study other obligate parasites of roots such as *Plasmodiophora brassicae, Polymyxa betae* (11,13), *Glomus mossae,* and *Gigaspora margarita* (12). When *A. rhizogenes* infects a wounded plant, a fragment of plasmid DNA from the bacterium is inserted into the plant genome. Transformed roots differ morphologically and physiologically from normal roots and are better adapted to grow in axenic culture (20). Transformed roots grow faster, are highly branched, and tend to grow horizontally instead of downward. Their high growth rate makes them effectively self-disinfecting, since they outgrow the bacterium (19).

The objective of this study was to determine if *Meloidogyne javanica* would reproduce on *A. rhizogenes*-transformed root cultures. Different plant species and stages and levels of nematode inoculum were compared.

Materials and Methods

Reproduction of M. javanica on transformed roots and root characteristics: Roots genetically transformed by *A. rhizogenes* strain A4 and free of bacteria were grown on Gamborg's B5 medium (GIBCO, Grand Island, New York) (2) plus vitamins (composition in milligrams per liter: calcium pantothenate, 1; nicotinic acid, 1; pyridoxine HCl, 1; thiamine HCl, 1; inosytol, 10; biotin, 0.01). The vitamins were filter (0.22-μm-pore) sterilized. Transformed root cultures included potato (*Solanum tuberosum* L. cv. unknown), bindweed (*Convolvulus sepium* L.), tomato (*Lycopersicon esculentum* Mill. cv. Tropic and cv. South Australian Early Dwarf Red [SAEDR]), carrot (*Daucus carota* L. cv. unknown), and lima bean (*Phaseolus lunatus* L., breeding line L 126). Root pieces (2–3 cm long) from actively growing cultures were transferred to new medium

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TABLE 1. Reproduction of *Meloidogyne javanica* on genetically transformed roots of several hosts cultured on artificial medium when inoculated with 72 ± 8 eggs per plate.

Host	Viable eggs†	Hatch (%)	Root wt (mg)	Egg masses	Females	Eggs/ plate	Eggs/g root	Pf/Pi	Infection‡
Potato	53	73	987 c	16 a	22 a	4,312 b	4,174 a	83 ab	43 b
Bindweed	48	63	712 dc	11 b	21 a	1,287 c	2,970 a	27 c	44 ab
Tropic tomato	38	58	2,450 a	11 b	12 b	1,805 c	747 b	48 bc	33 b
SAEDR tomato	44	59	1,667 b	20 a	24 a	6,795 a	4,218 a	161 a	56 a
Bean	52	70	1,895 ab	7 b	8 b	1,148 c	816 b	28 c	13 c
Carrot	43	63	220 d	1 c	2 c	61 c	726 b	2 d	2 c

Means are the average of six replications. Means in a column followed by the same letter are not different according to Duncan's multiple-range test, $P = 0.05$.

† Number of eggs (inoculum) that hatched.

‡ Percentage of viable eggs that reached adult female stage.

and incubated for 3 or 4 days before inoculation with nematodes.

Transformed root cultures were inoculated with 72 ± 8 eggs per plate. Eggs were obtained from monoxenic culture of the nematode on tomato root explants. Dispersed eggs were collected from egg masses incubated for 4 minutes in 0.5 ml 0.525% NaOCl in a sterile conical centrifuge tube. The egg suspension was then diluted 20 times with sterile distilled water. Eggs that settled within 30 minutes were used as inoculum (modification of Loewenberg et al. [9]). Six replicated plates of each root culture were prepared and incubated in the dark at 25 C. After 39 days, the agar was melted in a microwave oven, roots were removed, and fresh weight was determined. Females were dissected from the roots and counted. Egg masses were treated with 0.525% NaOCl for 5–10 minutes, and eggs were counted.

Eggs from tomato and potato root cultures were added to four UC82 tomato plants growing in autoclaved soil in a greenhouse (2,000 eggs/250 cm^3 soil). Root systems were examined for galls and egg masses after 60 days.

Nematode stage and inoculum level: Potato root cultures were started by transferring three root pieces to each of several plates. *M. javanica* eggs, egg masses, and juveniles were obtained from monoxenic culture of the nematode. Eggs were dispersed as described in the reproduction study. To obtain juveniles, several egg masses were placed in 2 ml sterile distilled water in a sterile vial, and nematodes that hatched within 48 hours were used. Juveniles were not surface sterilized. Egg masses were transferred directly from cultures. Egg masses (1, 2, or 4), dispersed eggs (83 ± 6, 171 ± 7, or 373 ± 34), or juveniles (42 ± 8, 80 ± 2, or 149 ± 17) ($\bar{x}$ ± SD) were aseptically transferred. Each treatment was replicated five times. Dispersed eggs, egg masses, or juveniles were added 2, 7, and 10 days after root transfer, respectively. Root fresh weight, number of females, egg masses, and eggs per plate were determined after incubation for 42 days in the dark at 25 C. Galls containing more than one female were termed "coalesced galls." The ratio between the final (Pf) and initial population (Pi) was calculated by dividing the number of eggs produced per plate by the number of juveniles or eggs that were added. Dispersed egg inoculum that did not hatch was not included in the Pi.

Data were transformed (log or arcsine) and analyzed by the general linear model procedure, and means were compared by Duncan's multiple-range test where indicated (17).

RESULTS

Reproduction of M. javanica on transformed roots and root characteristics: M. javanica reproduced on transformed root cultures of all plants tested; however, more females and eggs were obtained on potato and SAEDR tomato than on the other cultures

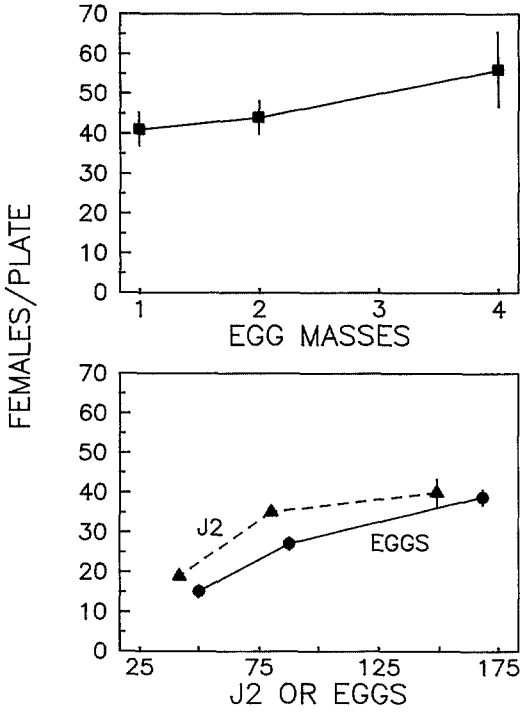

FIG. 1. Number of *Meloidogyne javanica* females produced on transformed potato root cultures as affected by stage and level of nematode inoculum. Egg inoculum is expressed as number of eggs hatching after addition to culture. Bars represent ± SEM. Bars smaller than the data symbols do not appear.

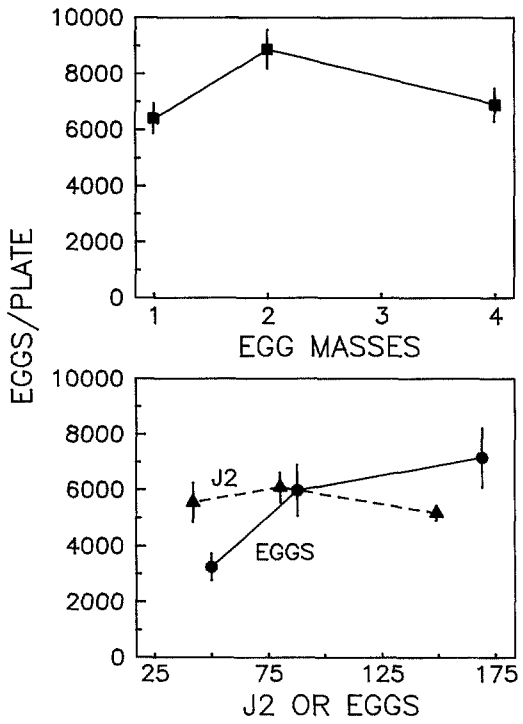

FIG. 2. Number of *Meloidogyne javanica* eggs per plate on transformed potato root cultures as affected by stage and level of nematode inoculum. Egg inoculum is expressed as number of eggs hatching after addition to culture. Bars represent ± SEM.

(Table 1). Roots that established quickly but subsequently grew at a moderate rate (potato and SAEDR tomato) supported the highest reproduction. Roots that grew very fast (Tropic tomato) supported lower reproduction. Transformed lima bean roots had few root hairs, were thick and tough, and produced fewer females and eggs. Although bindweed cultures supported high levels of females, egg production was low (Table 1). Transformed carrot roots grew very poorly on Gamborg's B5 medium; root growth ceased after a week, and nematode reproduction was very low.

More galls formed on roots in the agar than on the agar surface. SAEDR tomato tended to produce more roots below the surface than did the other cultures.

Galls and egg masses were observed on tomato plants grown in soil infested with *M. javanica* eggs from transformed root cultures of potato or both cultivars of tomato. Quantitative data were not collected.

Nematode stage and inoculum level: Number of females produced per plate increased with the amount of dispersed egg ($P < 0.01$) or juvenile ($P < 0.05$) inoculum, but this trend was not significant ($P > 0.05$) with egg mass inoculum (Fig. 1). Since only 60% of the dispersed eggs hatched, inoculum levels of eggs and juveniles were similar (in Figs. 1–3, egg inoculum is expressed as number of added eggs that hatched). The highest level of viable egg or juvenile inoculum (about 160) produced about the same number of females as did one egg mass (Fig. 1). The number of females or eggs produced in cultures was not proportional to the number of nematodes added (Figs. 1, 2).

The ratio between the final and initial population decreased sharply as the juvenile ($P < 0.01$) but not dispersed egg ($P >$

TABLE 2. Reproduction of *Meloidogyne javanica* on genetically transformed potato root cultures as affected by stage and level of nematode inoculum.

Inoculum	Level	Root wt (mg)	Fertile females†	Eggs/egg mass	Eggs/g root	Pf/Pi	Infection‡
Juveniles	42 ± 8	581 ± 191	80 ± 14	356 ± 105	11,000 ± 6,839	132 ± 67	45 ± 11
	80 ± 2	584 ± 136	79 ± 16	223 ± 38	10,777 ± 3,255	77 ± 25	44 ± 8
	149 ± 17	720 ± 376	60 ± 12	240 ± 47	9,419 ± 4,478	36 ± 10	27 ± 12
Viable eggs	50 ± 8	414 ± 147	85 ± 12	256 ± 46	7,999 ± 2,728	62 ± 24	30 ± 10
	87 ± 14	605 ± 345	69 ± 23	280 ± 54	13,289 ± 6,239	76 ± 53	31 ± 14
	168 ± 15	618 ± 122	70 ± 17	244 ± 52	12,418 ± 7,842	43 ± 22	23 ± 6
Egg masses	1	852 ± 355	79 ± 8	203 ± 42	10,746 ± 8,084		
	2	502 ± 219	82 ± 12	260 ± 69	21,314 ± 11,115		
	4	415 ± 200	63 ± 7	245 ± 101	21,596 ± 12,110		

Values are means of five replications ± standard deviation.
† Percentage of females that produced eggs.
‡ Percentage of nematodes that reached the adult female stage.

0.05) inoculum increased (Table 2). Higher inoculum levels did not result in an increase ($P > 0.05$) in eggs per plate (Fig. 2). In fact, eggs per female decreased sharply ($P < 0.05$) with increased juvenile inoculum (Fig. 3). Forty-three percent of the females were infertile when 149 juveniles were added. The percentage of nematodes infecting the roots (equals the number of nematodes that reached the adult female stage) was less at the highest inoculum level of juveniles and dispersed eggs than at lower inoculum levels (Table 2).

Females occurred in coalesced galls at the highest inoculum level of juveniles but not at lower inoculum levels. Such galls were observed less frequently when dispersed eggs were used as inoculum. Coalesced galls also occurred in cultures inoculated with two or four egg masses; galled tissue was necrotic and callused, and females in such galls were markedly small and distorted. Low numbers of males were sometimes observed in plates inoculated with four egg masses; males were not observed in any other treatments.

The hatching rate was higher (60%) when the dispersed egg Pi was 83 than when the dispersed egg Pi was 373 (45%).

DISCUSSION

Meloidogyne javanica was successfully cultured on several root cultures that had been genetically transformed by *A. rhizogenes*. The highest levels of nematode reproduction were obtained on transformed SAEDR tomato and potato. The superior performance of these cultures may be due to their growth habit. All other variables being equal, thin roots that grow at moderate rates into the agar and produce many secondary roots appear to support high nematode reproduction. Location of roots also appeared to affect nematode penetration and reproduction. More galls were observed on roots in the agar than on the agar surface.

The ingredients and their concentration in the culture medium greatly affect root and nematode development. The original formulation of Murashige and Skoog medium (14) inhibited giant cell formation by root-knot nematode. The inhibition was attributed to the high concentration of NH_4NO_3 in that medium (15). The concentration of nitrogen also influenced development of vesicular-arbuscular mycorrhiza fungal infection. Germ tubes stopped growing if total nitrogen concentration was above 2 mM (12). *M. javanica* infected and reproduced on transformed root cultures on a modification of the Murashige and Skoog medium (12) in previous experiments (21). In the present study, Gamborg's B5 medium plus vitamins in which all the nitrogen was supplied as KNO_3 was used. It has given good results culturing most endoparasitic nematodes (16). The

addition of vitamins to autoclaved medium enhanced the early establishment of the roots in the culture and apparently contributed to their active development.

Transformed roots were preferred over root explants (from germinated seeds) for monoxenic culture of *Meloidogyne* spp. for three reasons: First, transformed roots were easy to subculture by root transfer. They had been maintained in the laboratory for more than a year without any obvious decline in growth rate or change in characteristics. Problems associated with surface sterilization and germination of seeds were eliminated. Second, transformed roots developed more lateral roots and thus provided more infection sites for root-knot and other nematodes that infect just behind the root tip. Transformed SAEDR tomato cultures, which are highly branched, supported the highest level of *M. javanica* reproduction. Finally, more consistent results were obtained with transformed roots than with nontransformed roots. In early experiments, *M. javanica* reproduction was compared on transformed roots and on tomato root explants. The first nematode generation yielded much lower numbers of females and egg masses on root explants. Many juveniles were attracted to only a few root tips, resulting in the death of those root tips. More root tips of excised roots than of transformed roots were killed by overinvasion by the nematodes (Verdejo, unpubl.). Reasonable numbers of females and eggs were produced consistently after one generation on transformed roots, but comparable reproduction on root explants required two generations (1).

The selection of inoculum depends on the experiment to be performed. Egg masses, dispersed eggs, and juveniles all have advantages and disadvantages. The second-stage juvenile has the advantage that viability and number of juveniles are easily assessed; also, root penetration and infection are more synchronous than with egg masses or eggs. Juvenile inoculum should have been hatched less than 48 hours for highest levels of infection (Verdejo, unpubl.). In tissue culture studies,

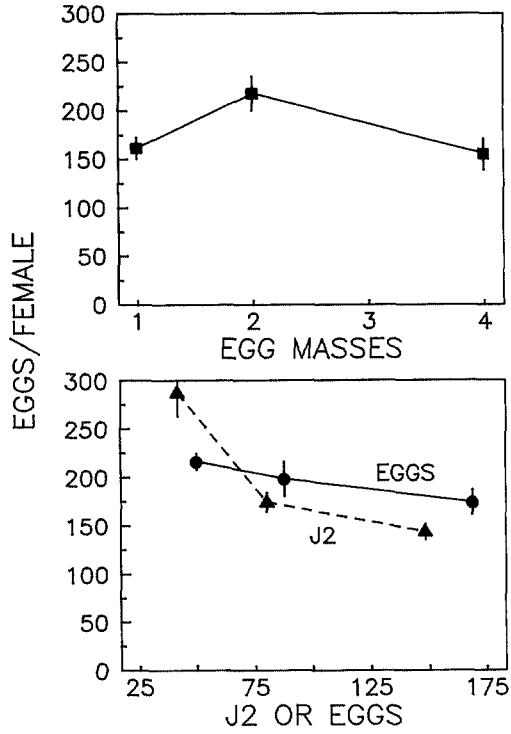

FIG. 3. Number of *Meloidogyne javanica* eggs per female (with or without egg masses) on transformed potato root cultures as affected by stage and level of nematode inoculum. Egg inoculum is expressed as number of eggs hatching after addition to culture. Bars represent ± SEM.

Meloidogyne spp. have been commonly introduced as egg masses (3,4,15,18). Egg masses are easily transferred and several hundred reproductive units are transferred at once. Quantification of inoculum is not possible, however, because the number of eggs per egg mass is highly variable. Dispersed eggs are easily quantified, and hatching and invasion take place gradually. Inconsistent results may occur because the hatching rate is unpredictable. In this study, an attempt was made to obtain healthy, viable eggs by using young egg masses. Hatching rate was higher when young egg masses were used, but a mixture of eggs in different developmental stages was always obtained.

There were more females per plate when the inoculum was increased. The results suggest, however, that intraspecific competition increased with higher inoculum.

Particularly with J2 inoculum, higher inoculum levels resulted in more coalesced galls and fewer eggs per female. The decrease in eggs per female resulted largely from an increase in the percentage of infertile females rather than a decrease in the number of eggs per fertile female (Table 2).

Monoxenic cultures of *Meloidogyne* spp. can be established on roots containing root-inducing, transferred DNA of *A. rhizogenes*. *M. javanica, M. incognita,* and *M. chitwoodi* are routinely maintained on transformed root cultures in our laboratory. Such cultures may be useful to study interactions of nematodes with other soil organisms. For example, transformed root cultures are currently being used to study the parasitism of *M. javanica* by *Pasteuria penetrans* (21).

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