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Transport of the Nematicide Oxamyl in Roots Transformed with *Agrobacterium rhizogenes*

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Abstract: Infection of roots transformed with *Agrobacterium rhizogenes* by *Meloidogyne incognita* and *Heterodera schachtii* second-stage juveniles was established in bicompartmental petri dishes. One compartment contained the Murashige and Skoog agar medium and the nematicide oxamyl, and the other compartment contained water agar. Transformed roots of carrot, tomato, alfalfa, cowpea, rape, and sugarbeet were placed in the nutrient compartment and grew over the barrier that divided the petri dishes and into the water agar compartment where juveniles were inoculated. The infective juveniles that thrust their stylets repeatedly into the apical cells of oxamyl-treated roots became immobilized. A comparison with previous studies on intact plants indicated that oxamyl was transported into the root tissues and diffused in the exudates.

Key words: Agrobacterium rhizogenes, Heterodera schachtii, Meloidogyne incognita, oxamyl, transformed root.

Various plant tissues, including seedlings, excised roots, and callus cultures, have been used to investigate plant–nematode interactions. Successful dual culture of plant tissues and nematodes has proved difficult for some nematode species. Phytoparasitic nematodes can be divided into migratory ectoparasites and endoparasites and sedentary endoparasites (23). Species of migratory ectoparasites and endoparasites (*Trichodorus* spp., *Tylenchorhynchus* spp., *Ditylenchus* spp., *Pratylenchus* spp.) can be maintained in vitro on suitable plant callus tissues (11). Summaries on nematode culture techniques under axenic conditions have been published (10,22,29). In contrast to the migratory ectoparasites and endoparasites, sedentary endoparasites (*Heterodera* spp., *Meloidogyne* spp., *Globodera* spp.) cannot be maintained as yet on undifferentiated callus tissues. Several reports described dual cultures with excised roots; in one instance (11), juveniles of *Meloidogyne incognita* (Kofoid & White) Chitwood penetrated and developed in roots of tomato grown on White's (27) medium supplemented with auxin and kinetin. Similar experiments have been performed with *Heterodera schachtii* Schmidt on excised roots of sugarbeet (11); however, the growth potential of excised roots was rather small, and the technique proved unsatisfactory for long-term maintenance of nematode cultures.

We suggest the use of dual cultures of *M. incognita* and *H. schachtii* and roots of their hosts containing the root-inducing (Ri) transferred-DNA (T-DNA) of *Agrobacterium rhizogenes* Riker. When *A. rhizogenes* infects a plant, part of the bacterial DNA (T-DNA) from the Ri plasmid is transferred to plant cells (6). Plant cells that receive the T-DNA and incorporate it into their genome differentiate into root meristems, and the roots grow rapidly without added phytohormones. The transformed roots are cultured in solid or liquid media (25). They have been used for the establishment of dual cultures of the biotrophic vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* Gerdemann & Trappe (15) and Plasmodiophorales (16).

In the present report, transformed roots of carrot, tomato, alfalfa, cowpea, rape, and sugarbeet were used to study the systemic nematicide–insecticide oxamyl. Oxamyl is nonphytotoxic and can be taken up by and translocated within the plant (12,13,21). The effect of oxamyl within transformed roots on the movement of second-stage juveniles (J2) of *M. incognita* and *H. schachtii* during the early stages of their development was studied. The use of this system was compared with studies on intact plants treated with oxamyl.

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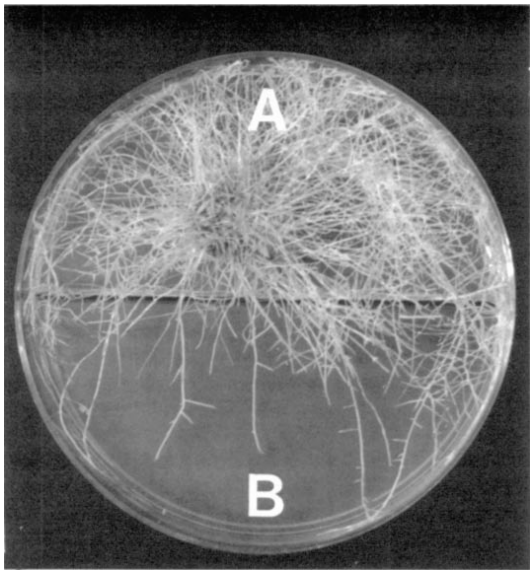

FIG. 1. Ten days growth of roots of carrot transformed with *Agrobacterium rhizogenes* in a bicompartmental 9-cm petri dish. Compartment A contains the Murashige and Skoog Difco agar medium and oxamyl (except controls); compartment B contains water Difco agar and juveniles of *Meloidogyne incognita*.

MATERIALS AND METHODS

Transformed root organ culture: Roots induced by *A. rhizogenes*, strain A_4 (ATCC no. 31,798), according to the procedures previously described (1,14–16,25) were established for alfalfa (*Medicago sativa* L.), cowpea (*Vigna unguiculata* Walp), rape (*Brassica napus* L.), tomato (*Lycopersicon esculentum* Mill.), and sugarbeet (*Beta vulgaris* L.). Thereafter, the transformed roots were maintained in a modified Murashige and Skoog (M&S) liquid medium containing 0.33 g/liter NH_4NO_3 and 0.38 g/liter KNO_3 (17).

Bicompartmental culture system: The transformed roots were cultured in divided petri dishes. The method, previously described (15), is illustrated in Figure 1. Compartment A contained 15 ml of 1.5% Difco agar M&S medium and compartment B contained 2% water Difco agar. Roots placed on the nutrient agar in compartment A began to grow into compartment B a few days after incubation at room temperature (22–25 C).

Oxamyl, which is a nonvolatile, water soluble, systemic nematicide active against several nematodes in various host plants (21), was tested at the following concentrations: 0, 5, 10, 100, 500, 1,000 mg a.i./liter. Oxamyl was diluted in sterile water, resuspended into the M&S agar medium at 40 C, and then poured into compartment A of each petri dish. Roots were placed on the oxamyl medium and were incubated for 1 week at 25 C in the dark. After this incubation period, the roots grew on the water agar in compartment B without any observable phytotoxic effect.

Nematode isolates: Meloidogyne incognita was reared on Marmande tomato and *Heterodera schachtii* was reared on Monosvalof sugarbeet, each growing in a substrate containing 50% loam and 50% sand mixture in plastic pots and kept for 1–2 months at 20–25 C with a 16-hour light and 8-hour dark cycle.

To obtain nematode inoculum, the infected plant roots were washed in running tap water until they were freed from soil particles. Egg masses of *M. incognita* and cysts of *H. schachtii* were excised from roots under a dissecting microscope and carefully cleaned. All nematodes with soil debris still adhering to them were discarded. Selected cysts and egg masses were agitated and repeatedly rinsed in sterile water. This procedure broke open the egg masses. The *H. schachtii* eggs were liberated from cysts by grinding in a blender. The eggs were washed onto sieves with at least six repeated rinsings in sterile water. Treatment of *H. schachtii* eggs in a 0.2% $ZnCl_2$ solution increased hatch (5). One milliliter of the nematode suspensions containing numerous eggs and hatched J2 (ca. 250 active J2/ml) were poured onto the agar in compartment B. The root cultures in compartment B were well established before the nematodes were added to provide close contact of J2 with the roots and their exudates. After inoculation, the movement of nematodes in compartment B was followed in situ by direct microscopic observations over a 5-day period. Eggs hatched during this period, giving the total of 350–400 active J2 per petri dish. Nematodes

within root tissues were stained by incubation in 0.05% trypan blue in hot lactophenol for 2–3 minutes.

Experiments with oxamyl in the bicompartmental culture system were replicated six times. The results were consistently reproduced in other experiments when we compared oxamyl with other nematicides.

Analytical method for oxamyl in exudates: The transformed roots were cultured in bicompartmental petri dishes. Compartment A contained the M&S agar medium and oxamyl (1,000 mg a.i./liter), and compartment B contained 10 ml distilled water. Following incubation in the dark, for 4 and 6 days at 25 C, the contents in compartment B were removed, diluted (v:v) in acetonitrile, and filtered through a 0.2-μm membrane. Oxamyl was analyzed by reverse phase high performance liquid chromatography using ultraviolet. The detection limit was 0.2 mg/liter (9).

Results

Nematode chemotaxis: Approximately 90–95% of the *M. incognita* J2 migrating along the agar surface reached the transformed carrot and tomato roots after 6–12 hours and were concentrated at the growing root tips. In contrast, none reached the roots of the nonhost alfalfa or cowpea. *Heterodera schachtii* J2 were not attracted to the transformed roots of the nonhosts alfalfa, cowpea, carrot, or tomato roots, but they showed a strong taxic response to the roots of the susceptible hosts, rape and sugarbeet. Infective juveniles congregated primarily at the root tips and the zone of elongation behind root tips. The J2 entered a susceptible root behind the growing tip or where a lateral root emerged. Upon penetration of carrot tissues by a single *M. incognita* J2, all other J2 that entered this root were observed to migrate away from the site of initial infection. This change in taxic response by *M. incognita* J2 was observed in all experiments performed with carrot roots. The *M. incognita* juvenile increased in size and growth, and giant cells were observed near the pericycle in the root cortex of carrot.

Table 1. Effect of oxamyl on *Meloidogyne incognita* and *Heterodera schachtii* second-stage juveniles growing in bicompartmental petri dishes containing roots of carrot and rape, respectively, transformed with *Agrobacterium rhizogenes*.

Oxamyl† (mg a.i./liter)	Juvenile migration (%)‡	
	M. incognita	*H. schachtii*
0	99.2 ± 1.2	90.9 ± 2.3
5	98.1 ± 4.9	82.4 ± 7.7
10	24.6 ± 10.3	27.4 ± 10.7
100	1.3 ± 0.7	0.8 ± 0.8
1,000	1.6 ± 0.9	1.1 ± 0.7

† Oxamyl uptake period was 1 week in compartment A which contained Murashige and Skoog Difco agar medium. After this period, the juveniles were inoculated on water agar in compartment B.

‡ Inoculum level (initial active J2 plus the newly hatched J2 during the infection period) was estimated at 350–400 second-stage juveniles (J2) per petri dish. The percentage of active J2 migrating on water agar in cmopartment B was estimated 2 days after inoculation.

Each value is the mean of six replicates; ± = SD.

Effect of oxamyl: Two days after inoculation, only 20–25% of the *M. incognita* J2 remained active in the presence of 10 mg oxamyl, compared with 100% in the controls (Table 1). At 100 mg oxamyl, nearly all the juveniles in the immediate proximity of the carrot and tomato roots were immobilized. They adopted various positions with some being coiled and others straight. The few (1.3%) that remained active were not attracted by the roots. Before dying, infective J2 positioned themselves close to a root tip and repeatedly thrust their stylets into the root epidermal cells. Dying J2 remained adjacent to the root tip but failed to penetrate the roots.

Heterodera schachtii J2 acted in the same way, attempting to penetrate rape and sugarbeet roots treated with oxamyl (Table 1). In the presence of the nonhost roots of cowpea and alfalfa that were treated with oxamyl, all *H. schachtii* J2 remained active over the 5-day observation period. They became less active, however, when their food reserves were depleted. When the nonhost roots were treated with 1,000 mg oxamyl, the J2 moved over the agar and crossed the roots without any change of direction or change in their pattern of activity. In contrast, those that sought a po-

TABLE 2. Release of oxamyl in root exudates and from roots of carrot and cowpea transformed with *Agrobacterium rhizogenes* growing in bicompartmental petri dishes.

Incubation period (days)	Exudates (mg/liter)	Roots (μg/mg)
	Carrot	
4	3.9	21.6
6	4.3	19.7
	Cowpea	
4	8.4	48.8
6	9.7	42.2

Compartment A contained Murashige and Skoog agar medium and oxamyl at 1,000 mg a.i./liter and compartment B contained 10 ml distilled water. Root links between the two compartments were cut, and the exudates and roots (fresh weight) in compartment B were analyzed for oxamyl. Each value is the mean of two replicates.

sition close to the susceptible roots and thrust their stylets repeatedly into oxamyl-treated root cells became immobilized.

Uptake and transport of oxamyl: Oxamyl moved into transformed roots (Table 2). The compound was absorbed by the roots in compartment A and translocated throughout the root tissue growing in compartment B. Approximately 9 mg/liter and 4 mg/liter oxamyl was recovered in cowpea and carrot root exudates, respectively. Although a relatively large amount (1,000 mg a.i./liter) of oxamyl was placed in compartment A, there was little oxamyl in the exudates and root tissue (Table 2) of cowpea or carrot (42–49 and 20–22 μg/mg tissue, respectively) after uptake periods of 4 and 6 days.

DISCUSSION

Although there is some evidence that the nematicide oxamyl is ambimobile, i.e., moving both apoplastically and symplastically (26), the way in which plants are protected by oxamyl from nematode invasion is not clear. Several authors (3,7,24) suggested that plants treated with foliar application of oxamyl produced nematicidal root exudates. Oxamyl was reported to diffuse out of potato tuber tissue when the tissue was transferred to an oxamyl-free medium (26). These findings are seemingly in conflict with other studies (2,4,8,20,28), suggesting that control of nematodes following a foliar application of oxamyl was due solely to the presence of oxamyl within the roots. Only traces of radioactivity were found in the soil surrounding potato treated with ^{14}C oxamyl, even though relatively large quantities were present in the roots (19).

In our experimental system, the transformed roots produced exudates containing substance(s) that attracted nematodes, and oxamyl-treated roots transported and exuded the nematicide. The oxamyl concentration of 9 mg/liter present in exudates of nonhost roots of cowpea did not affect movement of *M. incognita* J2. These results differ from those obtained for *M. incognita* on oxamyl-treated agar where only 0.5 mg a.i./liter suppressed the activity of J2 (28). The transport of oxamyl in the root tips of susceptible plants might be one explanation for the higher efficiency of oxamyl against *M. incognita* and *H. schachtii* J2 that accumulated in this area. Conversely, oxamyl was not exuded by other parts of the roots in quantities sufficient to affect the movement of nematodes near nonhost roots. Autoradiography using roots in a bicompartmental system as described previously (15) could provide relatively direct confirmation of such movements. The method has been reported for ^{14}C sucrose within transformed roots in a previous study (15).

Studies of *M. incognita* and *H. schachtii* grown in excised tissue culture initiated from axenic seedlings using the technique of White (27) are limited. Excised tissue culture has not been used widely in investigations of root–nematode interactions, mainly because the growth potential of excised roots was rather small and the media used contained high concentrations of sugar and phytohormones that affected nematode behavior (10,11). A bicompartmental transformed root system can supply separate nutrition for the roots and the nematodes. We have obtained transformed roots of carrot showing structures resembling root-knot nematode galls that were due to hypertrophy of root cells in which adult females fed. Although galls were obtained, we have not established lines of infected

roots because the nematode did not reproduce well and its development was retarded. On the other hand, recent attempts to culture *H. schachtii* on transformed roots of sugarbeet (18) and *Globodera rostochiensis* Woll. and *G. pallida* Stone on transformed roots of potato, *M. incognita* on transformed roots of carrot and potato, and *Heterodera carotae* Jones on transformed roots of carrot (Mugniery, pers. comm.) were successful; therefore, the method may be effective and merits further study.

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