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A Method for Generating *Meloidogyne incognita* Males

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Abstract: A method for producing mass quantities of *Meloidogyne incognita* males free from other developmental stages was developed. Host plants were grown hydroponically to facilitate nematode harvest. Pruning stress was shown to cause a higher percentage of juveniles to develop as males vs. a no-stress control. Application of pruning stress in the first 48 hr post-inoculation was also shown to be more effective at driving male development than at later times.

Key words: hydroponic, males, *Meloidogyne incognita*, method, pruning stress.

Environmental factors have a profound influence on sex determination in root-knot nematode (RKN) species, including *Meloidogyne incognita* (Trianataphyllou, 1973). Under ideal conditions, feeding J2 develop into egg-laying females. However, conditions that cause stress in the host or otherwise limit resources available to the developing nematodes redirect juvenile development towards males. High nematode inoculum density (Davide and Triantaphyllou, 1967), the presence of resistance genes in the host (Moura et al., 1993), or damage to the host as might be inflicted by pruning or application of foliar herbicides (Davide and Triantaphyllou, 1968; Orion and Minz, 1971; Ganguly and Dasgupta, 1984) all have been identified as masculinizing influences. These studies further indicated that commitment to sexual fate primarily occurs in feeding J2 at the time of, or shortly after, giant cell initiation (Trianataphyllou, 1973). Apart from gonadal development, the morphology and behavior of J2, J3 and J4 destined to become males are generally similar to those of those which will become females, although there are likely subtle differences. However, the J4-to-male molt produces a motile vermiform adult very different from the sessile females. Males typically emigrate from the host root shortly after molting (Eisenback, 1985).

A hydroponic method for producing RKN juveniles has previously been developed (Lambert et al., 1992). Here we combine this method with the application of timed host stress to develop a simple, optimized and highly reproducible method for the production of male *M. incognita* free of contamination from other developmental stages and other organisms.

Materials and Methods

Meloidogyne incognita were maintained on tomato plants (*Lycopersicon esculentum* 'Rutgers large red') grown in sterilized sand in a growth chamber. Tap water used to water plants or make nutrient solutions was passed through a water purifier (The Water Exchange, West Bloomfield, MI) with a 1-µm particle filter and KDF (chlorine reduction via redox) and GAF (organic reduction via activated charcoal) cartridges. Eggs were harvested, surface sterilized and stored for 4 d at 16°C to establish synchrony prior to hatching, as previously described (Hussey and Barker, 1973).

As far as possible, infection of plants for male *M. incognita* production was undertaken under gnotobiotic conditions (Koenning and Barker, 1985). Each treatment was replicated a minimum of five times. Rutgers seeds were sterilized by washing for 10 min in 6% sodium hypochlorite, then for 2 min in 75% ethanol, and rinsed five times in sterile water. Four seeds were planted per 5-cm^3 cell in a 24-cell tray using a sterile 4:1 sand to soil mix. Seedlings were bottom watered daily to maintain field capacity and fertilized weekly with NPK 20:20:20. After 4 wk, each seedling was inoculated with 1,000 J2 at a 2-cm depth in five locations around the root base to achieve uniform inoculation of 5,000 J2/plant. Lower leaves of inoculated plants were pruned either immediately after inoculation, or 1, 2 or 5 d later with a sterile scalpel, in each case leaving only one apical leaf and the shoot apex.

Two weeks after inoculation, plants were transferred to a hydroponic growth system. Soil was washed from the roots with filtered/KDF/GAF water, and the stem was wrapped at the soil line with a foam plug covered in plastic wrap to prevent water soaking. A 2-cm-diam. hole was cut in the lid of a 500-ml plastic container through which the plant was carefully inserted until the foam plug filled the hole in the lid. The container was filled with 400 ml of 0.5X Hoagland's No. 2 salt solution. A 2-mm aquarium tube was inserted through a slit in the lid until it reached the bottom of the container, and the internal end was fitted with a pumice aerator. The containers were covered in aluminum foil to block light, and the nutrient solution was changed every 3 d. The plants were grown at 60% humidity at 25°C under artificial light with gentle aeration supplied by an electric air pump. Males were harvested every 4 d by filtering the hydroponic tank contents through a 250-µm-pore sieve stacked on a 43-µm-pore sieve. Nematodes collected on the 43-µm-pore sieve were examined by microscopy to confirm identity and counted.

Results and Discussion

Hydroponic culture medium was examined for the presence of nematodes that had emigrated from host

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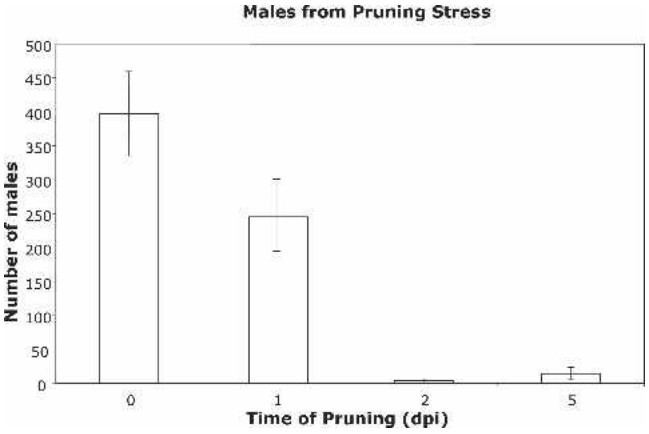

FIG. 1. Number of male nematodes recovered from hydroponically grown plants stressed by pruning at various days post infection (dpi). Bars indicate standard error.

roots beginning 18 dpi (days post-infection) and at regular intervals thereafter for up to 45 dpi. Unstressed plants yielded only nine male nematodes during this period, corresponding to only 0.18% of the J2 used for inoculum. By contrast, plants that been subjected to pruning stress at 1 or 2 dpi yielded large numbers of motile nematodes (Fig. 1), representing approximately 14% of the inoculum. Examination by stereomicroscopy revealed that the nematodes recovered were all *M. incognita* males; no other juvenile or adult stages were seen. These yields of males observed in our study are similar to those previously reported (Davide and Triantaphyllou, 1967) under nutrient deficiency host stress.

Males were found in collections made as late as 45 dpi, although approximately 90% of males were found in the collections made between 18 dpi and 30 dpi. These results confirm previous conclusions that not only does pruning stress induce male sexual development, but also that the response of this stress is limited to the first few days after infection (Trianataphyllou, 1973). Microscopic analysis revealed that at least 90% of the males generated by this method had both only one gonad and tail spicules, which are diagnostic characteristics of true males and consistent with evidence from previous studies (Papadopoulou and Triantaphyllou, 1982).

It has been postulated (Trianataphyllou, 1973) that the nature of the stress induced by pruning and perceived by *Meloidogyne* spp. closely resembles nutrient deficiency. In both conditions, the development of the J2 into an adult is slowed, and the male-to-female ratio is increased due to a switch in the developmental fate of the J2 rather than due to differential death of females. There are many potential physiological and biochemical differences between plants under nutrient deprivation and pruning stress, but the signal for RKN sex determination is apparently common to both conditions. It is still not clear whether this signal is part of a host regulatory pathway (conceivably a single molecule produced by the plant) or a more complex physiological cue perceived by the nematode as a measure of the availability or quality of nutrition, which we term "food signal." Genetic analysis (Lohar and Bird, 2003) has shown that the ability of RKN to establish successful feeding sites is modulated by the same shoot-derived signaling pathway that regulates the number of nitrogen-fixing nodules induced by rhizobacteria on legumes. However, that study did not reveal altered nematode sex ratios (Lohar and Bird, 2003). Thus, a more plausible hypothesis of the nature of the cue leading to male development is based on the notion of food signal. In *C. elegans*, development of the dauer larva to allow prolonged survival and environmental resistance is triggered by low levels of a general food signal relative to total nematode numbers, which is computed by the *C. elegans* L1 from the concentration of a constitutively secreted pheromone (Golden and Riddle, 1984). In fact, this ratio is measured twice during development, demonstrating the functionality of this developmental plasticity throughout development. *Meloidogyne incognita* J2 and *C. elegans* L2 both express glyoxylate pathway enzymes needed for lipid metabolism characteristic of nematode survival stages (McCarter et al., 2003). Reentry into the L3 stage from the dauer also is triggered by a general food signal, in this case the reappearance of a relatively adequate food source. Recent evidence demonstrates that *C. elegans* L2 also respond to a general food signal for sex determination, where more males develop when grown in log-phase vs. stationary-phase *E. coli* (Prahlad et al., 2003). Understanding the basis for this decision might reveal a mechanism widely used across the phylum.

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