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Esterase Polymorphism in *Meloidogyne konaensis*

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Abstract: The continual detection of a slow (I1) esterase band in greenhouse cultures of *Meloidogyne konaensis* isolated from the field led to a hypothesis that the nematode may be polymorphic for esterase. A survey of coffee fields demonstrated at least four esterase phenotypes were present in *Meloidogyne* recovered. An F1 phenotype predominated (60% of the females), but an I1 phenotype was also common (30% of samples). A series of greenhouse and laboratory experiments were undertaken to understand this polymorphism. Esterase phenotype was not affected by development at 22°, 25°, or 33 °C on tomato. Two different esterase phenotypes (I1 and F1-I1) were detected after *M. konaensis* was grown on tomato for several generations, even in single-egg-mass lines derived from an F1 female. Three isolates of *M. konaensis* differing in esterase phenotype (F1, I1, and F1-I1) did not differ morphologically but did differ in their parasitic ability. Only the F1 isolate parasitized *Coffea arabica*. The F1-I1 isolate had greater reproduction on *Lycopersicon esculentum* and *Cucumis sativus* than either the I1 or F1 isolate. The mechanism of the development of the polymorphism has yet to be determined. However, the F1 esterase may be useful as a marker for future research on parasitism of coffee by *M. konaensis*.

Key words: coffee, esterase polymorphism, genetic variation, host range, *Meloidogyne konaensis,* parasitism, root-knot nematode, selection.

While root-knot nematodes had been diagnosed on coffee for many years in Hawaii, only limited research had been conducted by the late 1980s. We collected a *Meloidogyne* species infecting coffee in 1990 and established cultures on *Lycopersicon esculentum* cv. Orange Pixie in the University of Hawaii at Manoa greenhouses for further research. The nematode was described as a new species, *Meloidogyne konaensis,* the Kona coffee root-knot nematode, in 1994 (Eisenback et al.). *Meloidogyne konaensis* is one of several nematode species that parasitize *Coffea arabica.* Like many of the root-knot nematodes parasitizing *C. arabica, M. konaensis* has an F1 esterase phenotype (Eisenback et al., 1994).

In 1993, our isolate of *M. konaensis* had been maintained in the greenhouse for 18 to 24 months. These isolates were regularly subcultured by extracting eggs from infected tomato roots (Hussey and Barker, 1973) and reinoculating these eggs onto tomato plants. Pots containing *M. konaensis* were regularly assayed for possible contamination by other root-knot nematode species using esterase phenotype. During a 1993 assay, isolates were found to contain only females with an I1 esterase phenotype. We concluded *M. incognita* contamination had displaced the *M. konaensis*. Consequently, a new field isolate of *M. konaensis* was collected and placed into the greenhouse. Unknown to us at the time, isolates shared with colleagues were found to contain nematodes with multiple esterase phenotypes and therefore were assumed to be contaminated (Caneiro et al., 1996a). In December 1995, as a series of new experiments was planned, *M. konaensis* isolates were assayed for their esterase phenotype. Again, the females assayed were all of the I1 esterase phenotype. We were unable to detect any females with the F1 esterase phenotype in additional assays of the nematodes in culture.

For a third time, field isolations of *M. konaensis* were collected to re-establish the cultures. Females from the first generation on tomato were assayed for esterase, and a mixture of I1 and F1 phenotypes were detected. Egg masses from those females with the F1 esterase phenotype were combined and inoculated onto tomato. In the following generation, females were assayed for their esterase phenotype and male stylet morphology was observed. Whereas males exhibited stylet projections as expected for *M. konaensis,* some females exhibited an I1 esterase phenotype. Consequently, single-egg-mass lines were established from individual F1 esterase females. These isolates were separated and maintained in a greenhouse 40 km from other *Meloidogyne* isolates.

The continual contamination of *M. konaensis* isolates was suspicious. Considering we did not experience contamination among any other root-knot nematode isolates, we hypothesized that the appearance of the I1 esterase phenotype resulted from a genuine biological process. We undertook a series of experiments to further evaluate the esterase phenotype associated with *M. konaensis.* Our objectives were to (i) determine the effects of temperature on the esterase phenotype of *M. konaensis,* (ii) compare the morphology and parasitic ability of *M. konaensis* isolates differing in esterase phenotype, and (iii) determine the frequency of different esterase phenotypes in coffee fields where *Meloidogyne* was present.

Materials and Methods

Temperature effects: Eggs of *M. konaensis* were collected from cultures by shaking the tomato roots in 0.5% NaOCl (Hussey and Barker, 1973). Inoculum for the first repeat of the experiment was collected from toma-

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toes. Inoculum for the second repeat of the experiment was collected from coffee. In each repeat, six Orange Pixie tomato plants growing in 13-cm-diam. pots filled with a sterile 1:1 mixture of a clay soil and quartz sand were inoculated with 5,000 eggs of *M. konaensis.* Two inoculated plants were placed in growth rooms lit for 12 hours each 24-hour period set at temperatures of 22, 25, or 33 °C. Plants were watered daily and fertilized as needed.

Six weeks after inoculation, females were excised from the tomato roots, placed into 1.5-ml Eppendorf tubes filled with 0.5 ml 0.16 M saline, frozen at −20 °C, and electrophoresed 2–3 days later. At least 10 plump, pearly white females were handpicked from each plant. Polyacrylamide gel electrophoresis (PAGE) was performed using the Phastsystem (Amersham Pharmacia) on 10–15% gradient gels and native buffer strips. The system was cooled to a standby temperature of 5 °C. The *M. konaensis* females were defrosted, placed on ice, and individually transferred to wells containing 2.5 µl of extraction buffer (20% sucrose, 2% Triton X-100, and 0.01% bromophenol blue in water) (Esbenshade and Triantaphyllou, 1985). Individual females were macerated and the resulting solution drawn into an eight-well sample applicator. Two *M. javanica* females were used as a standard on each gel.

Following electrophoresis, gels were stained for esterase activity with 25 ml of 0.1 M pH 7.4 phosphate buffer, 7.5 µg EDTA, 15 µg Fast Blue RR salt, and 0.010 g α-naphthyl acetate in 0.5 ml of acetone (Esbenshade and Triantaphyllou, 1985). The stained gels were fixed overnight in a solution of 10% acetic acid and 10% glycerol. The bands were scored in relation to the mobility of esterase bands resolved for *M. javanica.*

Host selection: Esterase phenotypes of the *M. konaensis* F1 and I1 isolates from the temperature experiment mentioned above were assayed over the next two generations as the isolates were cultured on tomato. Five hundred eggs from each isolate were inoculated onto individual 6-week-old Orange Pixie tomato transplants growing in 13-cm-diam. clay pots filled with steam-sterilized soil. The plants were maintained in the greenhouse on a bench separate from other root-knot nematode isolates. Females and eggs were collected from the roots 45 days after inoculation. Up to 20 females were picked and frozen in saline as described above. Eggs were inoculated onto a new tomato plant. Females were crushed, electrophoresed, and scored for esterase phenotype as described above.

In a second experiment, six single-egg-mass lines from F1 esterase female *M. konaensis* progenitors were established and assayed for esterase phenotype over the next 20 months. Each progenitor female was tested to document her F1 esterase phenotype. The six single-egg-mass lines were established by inoculating an egg mass collected from a single female onto a 2-week-old Orange Pixie tomato. Seedlings were grown in 4-cm-diam., 29.5-cm-tall glass tubes filled with sterile quartz sand. The tomato plants were placed under lights on a laboratory bench. The plants were watered and fertilized by hand to prevent splash and possible tube-to-tube contamination. Seventy-five days after inoculation, plants were removed from the tubes, placed on nested 246- and 25-µm opening sieves, and gently washed in tap water to remove the sand and collect males. Any males recovered were mounted on a glass slide and their stylet morphology observed with a light microscope under oil immersion. The roots were shaken in a 0.05% NaOCl solution for 4 minutes to extract eggs. Females were picked from the roots, frozen in saline, and assayed for esterase phenotype as described in the temperature experiment. Eggs were reinoculated onto tomato transplanted into a sterile tube and the entire process repeated every 75 days.

Morphological comparison: The morphologies of male heads and stylets were examined from (i) a *M. konaensis* single-egg-mass line with a two-band esterase phenotype (MkF1-I1), (ii) a *M. konanensis* greenhouse isolate with a single fast-band esterase phenotype (MkF1), and (iii) an isolate of *Meloidogyne* collected from the Greenwell coffee plantation with a slow-band esterase phenotype (MspI1). The species description of *M. konaensis* was used as reference (Eisenback et al., 1994). Orange Pixie tomato plants, inoculated with eggs 30 days earlier, were removed from pots and washed with tap water to dislodge soil. Males were extracted from the soil by centrifugation and flotation (Jenkins, 1964). Twenty males were mounted on semipermenant slides and examined using light microscopy under oil immersion.

Parasitism: The parasitism and reproduction of three isolates of *M. konaensis* differing in esterase phenotypes (MkF1, MkF1-I1, and MkI1) were compared in a factorial design experiment on three different hosts (*C. arabica* cv. Typica, Selection 502; *L. esculentum* cv. Orange Pixie; and *Cucumis sativus* cv. Sweet Success). Each nematode-host combination was replicated four times, and the experiment was repeated once.

Seeds were germinated in vermiculite and transplanted into 10-cm-diam. clay pots filled with a sterilized 1:1 clay soil, silica sand mixture. Coffee and tomato seedlings were transplanted upon reaching the first true leaf stage. Cucumbers were transplanted 4 days after germination. In the first repeat of the experiment, coffee seedlings were inoculated 60 days after transplanting whereas in the repeat, coffee seedlings were inoculated 108 days after transplanting. Tomato and cucumber were inoculated 14 days after planting in each repeat. Inoculum from the three isolates was extracted by shaking infected roots in a 0.05% NaOCl solution (Hussey and Barker, 1973) and adjusting the suspension to give 600 eggs/ml. Each plant received 3,000 eggs in a 5-ml aliquot.

Plants were removed from the pots 60 days after inoculation. The shoots were cut at the soil line and

weighed. The roots were washed clean of soil and root wet weight recorded. Eggs were extracted by blending the roots in a 0.05% NaOCl solution (Hussey and Barker, 1973), collecting the eggs on a 20-μm mesh screen, and using centrifugal flotation to separate the eggs from plant debris (Jenkins, 1964). The shoots and roots were oven-dried at 70 °C for 2 days and weighed. The total number of J2 and eggs were added to obtain a final nematode population density (Pf). Reproduction ratios (Rf) were calculated by dividing Pf by 3,000. The number of eggs and J2 per g wet root was also calculated.

Analysis of variance of the two-way factorial experimental design (host × nematode esterase phenotype) was conducted (SAS Institute, Cary, NC). The data for the two repeats were tested for homogeneity of variance, found not different ($P > 0.05$), and combined for analysis of variance. The number of eggs and J2, and eggs and J2 per g wet root weight were $\log_{10}$ $(n + 1)$ transformed to normalize the data. Rf values were square-root transformed and analyzed for variance. The untransformed means are presented for clarity. A Waller-Duncan *k*-ratio *t* test was used to separate differences among treatments.

Field esterase phenotypes: Meloidogyne females from five coffee fields located in Kona, Hawaii, were assayed for esterase phenotypes. Soil from the drip line of the coffee trees was collected and weed roots removed. The soil was transferred to the laboratory, placed into 15-cm-diam. clay pots, and an Orange Pixie tomato transplanted into each pot. Four to 6 months after planting the tomato, root-knot nematode females were handpicked from the tomato roots, placed in saline solution, and frozen. Numbers of females assayed varied from field to field, but at least 10 females were assayed for each field. The esterase phenotype of each female was determined within 1 month of picking (Esbenshade and Triantaphyllou, 1985).

One Kealakekua, Hawaii field was sampled three times: in 1999, 2001, and 2002. In the 2002 sampling, 43 *Meloidogyne* females were handpicked from two *Amaranthus spinosus* growing as weeds in the field and frozen at −80 °C. Soil from around the coffee tree drip line was collected, placed into pots, and planted with a tomato. Six months after planting, 68 females were teased from tomato galls and assayed for esterase phenotype along with those collected earlier from amaranth.

Results

Temperature effects: Changes in temperature had no effect on the esterase phenotype of *M. konaensis* females (Fig. 1). In the first repeat (inoculum from tomato), females had a slow-band or double-band esterase phenotype at 22, 25, and 33 °C. In the second repeat (inoculum from coffee), females expressed a fast-band esterase phenotype at all three temperatures.

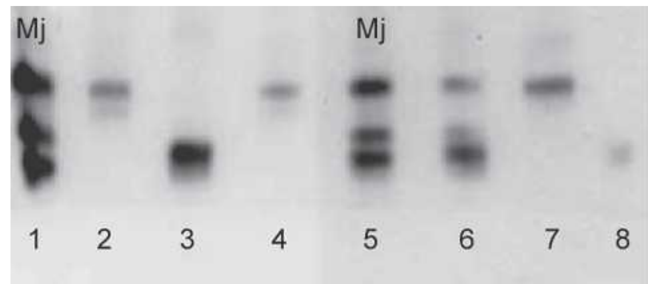

Fig. 1. Esterase phenotypes of *Meloidogyne konaensis* grown on *Lycopersicon esculentum* cv. Orange Pixie at 22, 25, and 33 °C. Lanes left to right: 1) *Meloidogyne javanica,* 2) slow-band female from tomato inoculum developing at 22 °C, 3) fast-band female from coffee inoculum developing at 22 °C, 4) slow-band female from tomato inoculum from tomato at 25 °C, 5) *M. javanica,* 6) double-band inoculum from coffee at 25 °C, 7) inoculum from tomato at 33 °C, and 8) inoculum from coffee at 33 °C.

Host selection: Cultivation of the F1 esterase *M. konaensis* on tomato selected for a different esterase phenotype (Fig. 2). The F1 esterase phenotype females showed the same phenotype in the second generation (host sequence = coffee-tomato-tomato). However, in the third generation of the F1 isolate (host sequence = coffee-tomato-tomato), we detected a slow band in one of 18 females assayed. In this experiment, no F1 females were detected in the I1 phenotype isolate. Because we assayed no more than 20 individuals, it would be difficult to detect changes that did not occur in frequencies greater than 5%.

Selection of esterase phenotypes was also detected in the single-egg-mass lines of *M. konaensis* grown on tomato in tubes. In the first- and second-generation assays, all females from all six lines expressed the F1 phenotype. In the third-generation assay, two females from one line expressed an I1 band. Ten other females from the same plant had F1 phenotypes. In generation four and five, all females assayed expressed the F1 phenotype. In generation six, one female with a double-banded esterase pattern was detected (Fig. 2). At this point, the nematodes were no longer reproducing well in the laboratory and three of the lines went extinct. By

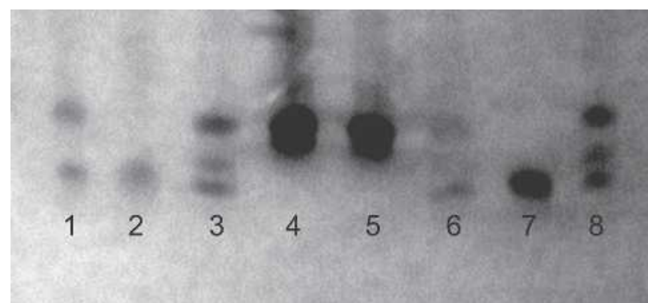

Fig. 2. Fast (F1), slow (I1), and double (F1-I1) esterase phenotypes of *Meloidogyne konaensis* females collected from tomato. Lanes left to right: 1) F1-I1 double band, 2) F1 fast band, 3) *Meloidogyne javanica,* 4) *Meloidogyne incognita,* 5) I1 slow band, 6) F1-I1 double band, 7) F1 fast band, and 8) *Meloidogyne javanica.*

generation seven, two of the remaining lines had gone extinct.

Males developed infrequently, often with only one or two recovered from each generation. However, those observed exhibited morphological features characteristic of *M. konaensis*. Three males were recovered in the third-generation assay, each from a different line. Two of the males fit the description of *M. konaensis* well, whereas the third male had a thinner stylet with longer knobs, no stylet projections, and a short, rounded tail. In generation five, males were recovered from all six lines and all fit the description for *M. konaensis*.

Morphological comparison: Differences observed among the male heads and stylets were not substantial. All MkF1 males had the typical high and rounded head cap (Fig. 3A,B). The head caps of the majority of MkF1-I1 were lower than those of MkF1 (Fig. 3C,D) but still similar to the *M. konaensis* description. For the MspI1 isolate, greater variation was observed in the male heads. Most MspI1 males (16 of 20) had head caps that were narrow, square, and indented anteriorly (Fig. 3E,F). Four of MspI1 males had head caps that were narrow but rounded. The stylet shafts of MkF1 males were not smooth. Two to four obvious projections were observed on all 20 MkF1 male stylets. The majority, 95%, of the MkF1-I1 male stylets had projections on the shaft. Nine of the MspI1 male stylets were smooth whereas 11 had projections. Overall, morphology of the males fit the description of *M. konaensis*.

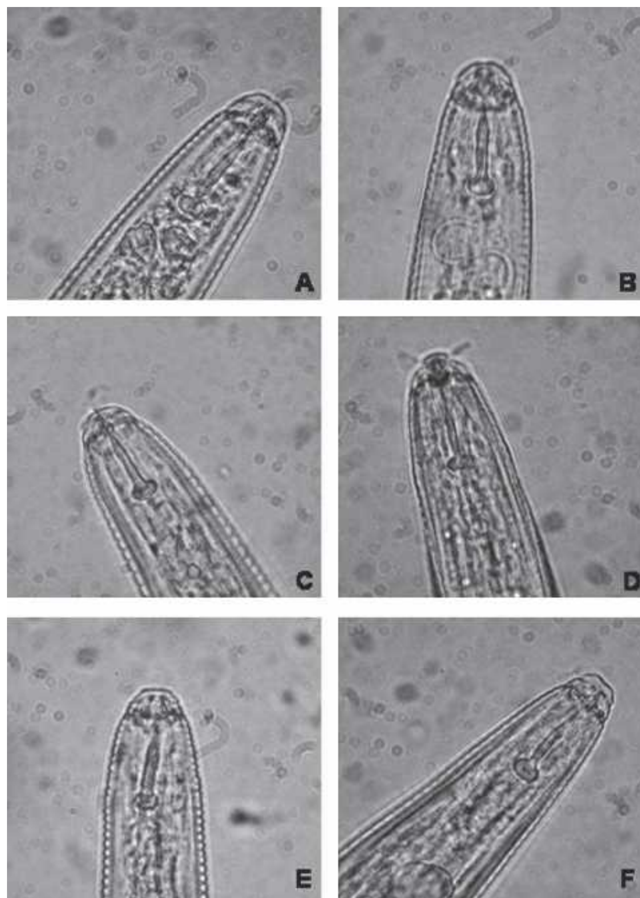

FIG. 3. Heads and stylets of males of *Meloidogyne konaensis* from parental lines with a single (MkF1) or a double-band esterase phenotype (MkF1-I1) and from a parental line of a female collected in a coffee field exhibiting an I1 esterase phenotype (MspI1). A, B) MkF1. C, D) MkF1-I1. E, F) MspI1.

Parasitism: Overall, tomato was the best host and coffee the poorest host for the three isolates. The average Rf of all nematodes on tomato was greater than on cucumber or coffee ($P < 0.05$). The number of eggs and J2 developing on tomato was 180 times greater than on coffee and three times greater than on cucumber. The same trend held for Rf and eggs and J2 per g wet root. Cucumber was also a good host for all three isolates (Table 1).

The MkF1-I1 isolate reproduced at the highest level among the three isolates. MkF1-I1 Rf was greater than the Rf of MkF1 and MkI1 (Table 1). The number of eggs and J2 of MkF1 and MkI1 were not different from each other ($P > 0.05$).

An interaction between host and nematode isolate occurred. Coffee was a host for MkF1 and a nonhost for MkF1-I1 and MkI1 (Table 1). The mean number of eggs and J2 on coffee was 3,402 for MkF1 and 0 for MkF1-I1 and MkI1. No isolate by host interaction was detected for wet and dry root and shoot weight. No differences in wet and dry root and shoot weight of tomato, cucumber, or coffee were found among three nematode isolates.

Field esterase phenotypes: Four esterase phenotypes were found in samples from the coffee fields in Kona and included the F1, F1-I1, and I1 phenotypes (Table 2). Most farms contained mixed phenotypes, with the F1 phenotype predominating. F1 phenotypes were observed with I1, F1-I1, and a four-banded phenotype on one farm. Overall, F1 females accounted for 60% of the nematodes recovered from the coffee fields. The I1 phenotype represented 30% of the females assayed, and the other esterase patterns combined represented only 10%.

The females collected from amaranth on the coffee

TABLE 1. The average number of eggs and second-stage juveniles (J2) per plant and mean reproduction ratio (Rf = Pf/Pi) of three isolates of *Meloidogyne konaensis* on *Lycopersicon esculentum* cv. Orange Pixie, *Cucumis sativus* cv. Sweet Success, and *Coffea arabica* cv. Typica, Selection 502.

	Tomato		Cucumber		Coffee	
Isolate	Eggs and J2[1]	Rf[1]	Eggs and J2	Rf	Eggs and J2	Rf
F1	137,800 b[2]	45.9 b	28,090 b	9.3 b	3,402 a	1.1 a
F1-I1	332,536 a	110.9 a	122,280 a	40.8 a	0 b	0 b
I1	142,933 b	47.6 b	52,818 b	17.6 b	0 b	0 b

[1] Data are means of eight replications. The egg and J2 data were $\log_{10}(n+1)$ transformed. Rf values were square-root transformed. The untransformed means are presented.

[2] Numbers in a column followed by the same letter are not different according to a Waller Duncan *k*-ratio *t* test (*k* ratio = 500).

TABLE 2. Frequency of different esterase phenotypes of *Meloidogyne* spp. found in the Kona, Hawaii area coffee fields surveyed between 1998 and 2002.

Locality	Females assayed	Esterase phenotype			
		F1	I1	F1-I1	Other
Captain Cook-1	10	-	1.00	-	-
Captain Cook-2	64	0.70	0.28	0.02	-
Holualoa	10	0.33	0.33	0.33	-
Honaunau	18	0.50	0.17	0.33	-
Kealakekua	39	0.53	0.34	-	0.13

farm exhibited 79% F1 and 21% I1 esterase phenotypes. Females recovered from tomato grown in the infested field soil from the same farm exhibited F1 (69%), F1-I1 (4%), and I1 (27%) phenotypes.

DISCUSSION

Females of *M. konaensis* are polymorphic for esterase. Whereas *M. konaensis* was originally described as possessing an F1 esterase phenotype (Eisenback et al., 1994), we have detected females that have a single slow band (I1) phenotype, similar to the I1 phenotype of *M. incognita* (Esbenshade and Triantaphyllou, 1985), or a double band (F1-I1) phenotype. These root-knot nematodes, while differing in esterase phenotype, had morphology that was consistent with a diagnosis of *M. konaensis.* All of these nematodes also had N1 malate dehydrogenase phenotypes (data not presented). Cultured isolates of *M. paranaensis,* described as having the F1 esterase phenotype, also express a slow and fast band esterase phenotype (J.-L. Sarah, pers. comm.). *Meloidogyne paranaensis* isolated from coffee was described previously and is closely related to *M. konaensis* (Carneiro et al., 1996b).

Multiple esterase phenotypes are not uncommon in *Meloidogyne* species. *Meloidogyne arenaria* has perhaps the most variation in esterase, with six different reported phenotypes (Esbenshade and Triantaphyllou, 1985). *Meloidogyne incognita* has three reported phenotypes (Carneiro et al., 2000; Esbenshade and Triantaphyllou, 1985). Three esterase phenotypes have been reported for *M. partityla* (Starr et al., 1996) and two for *M. hapla* (Esbenshade and Triantaphyllou, 1985). In coffee fields, the F1 phenotype of *M. konaensis* is most common. The I1 and F1-I1 phenotypes of *M. konaensis* also can be found growing on weeds within coffee fields but are uncommon. The inability of the I1 and F1-I1 phenotype females to develop on coffee probably excluded these phenotypes from the original collection and inclusion in the species description.

Many species of *Meloidogyne* that parasitize coffee express an F1 esterase phenotype (Esbenshade and Triantaphyllou, 1985). Eventhough the appearance of F1 esterase is not diagnostic for a particular species, this phenotype does appear to be associated with *M. konaensis* parasitism on coffee. Consequently, the F1 phenotype may serve as a useful genetic marker for the study parasitism on coffee by *M. konaensis.* Compared to other plants, coffee is a rather poor host to the F1 phenotype of *M. konaensis* (Zhang, 1994; Zhang and Schmitt, 1994). Yet, when coffee predominates as a host, the possibility of selection for coffee parasitism and the F1 esterase exists. In the presence of better hosts, the coffee parasitism genes may be selected against and the I1 and F1-I1 esterase phenotypes increase in frequency. The need for multiple generations to pass on hosts other than coffee for noncoffee-parasitizing phenotypes of *M. konaensis* to increase to detectable levels supports this scenario. Hypothetically, it may be possible to manage *M. konaensis* in coffee by growing tomato as cover crop and then, once assays demonstrate a population shift to the I1 or F1-I1 phenotype, replant coffee.

Whereas selection can explain the loss of the F1 phenotype of *M. konaensis* over multiple generations on tomato, it fails to explain how this polymorphism develops in a mitotically parthenogenic nematode. *Meloidogyne konaensis* is polyploid, 2n = 44 (Eisenback et al., 1994), therefore, it is reasonable to assume that duplicate esterase genes must exist in the nematode's genome. Changes at the F1 esterase locus may lead to the I1 esterase expression and the loss of coffee parasitism. Mutations, transposable elements, or environmental factors may be responsible for these changes (Fu et al., 1998; Navas et al., 2001). The frequency at which the I1 phenotype appears in F1 phenotype populations would represent an unusually high mutation rate. However, transposons are common in *Caenorhabditis elegans* (Fischer et al., 2003) and alter gene expression in other animals and plants (Kidwell and Lisch, 2000). It is possible that transposable elements active in *M. konaensis* are responsible for the differences in esterase phenotypes. Further molecular analysis is warranted.

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