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An In Vitro Test for Temperature Sensitivity and Resistance to *Meloidogyne incognita* in Tomato[1]

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Abstract: An in vitro root explant tissue culture technique is described for determining susceptibility of tomato (*Lycopersicon esculentum* Mill.) breeding lines and cultivars to the root-knot nematode *Meloidogyne incognita.* Root explants were taken from 2-day-old seedlings cultured for 30 days at 28 C on Gamborg's B-5 medium with or without nematode inoculum. The remaining portion of the root and stem from the excised root explants was transferred to soil in pots and grown to maturity in the greenhouse. In vitro root explants were evaluated for growth and occurrence of juveniles, adults, and egg masses. The regenerated plants were used to produce more seed. The proposed technique is simple, reliable, and adapted to routine screening of large numbers of F_1 and F_2 samples, and it utilizes less space than tests performed on intact plants in the greenhouse or growth chamber. Evidence is presented also on the breakdown of resistance to *M. incognita* under high temperature stress using this in vitro root explant technique.

Key words: Culture, *Lycopersicon esculentum, Meloidogyne incognita,* nematode, resistance, root-knot nematode, temperature.

In many areas worldwide, *Meloidogyne incognita* (Kofoid & White) Chitwood, the southern root-knot nematode, presents a major economic problem by inflicting serious losses that limit successful production of vegetable crops (22). Breeding programs for resistance to this nematode, especially for tomato (*Lycopersicon esculentum* Mill.), have been in existence for many years with the objective of identifying the various sources of resistance in wild and domestic species and introducing them into promising breeding lines and cultivars (21). As a result, all commercial cultivars resistant to *Meloidogyne* carry the *Mi* gene (17,18).

Even though resistant cultivars have been produced through traditional breeding programs, selection and testing are labor and space consuming. These screening methods require growing the plants in a growth chamber or a greenhouse, inoculating the intact seedling at a specific stage, and examining tissue after a certain period for infection (13,16). At present, screening for resistance to nematodes requires an appreciable amount of seeds to propagate a large number of test plants, and few available screening techniques are nondestructive in early phases of a breeding program (13).

Furthermore, screening tomato germplasm for resistance to root-knot nematodes is particularly important in breeding for heat tolerance. Nematode infections may weaken plants under heat stress and make the plants more subject to damage due to the nematodes (5), and heat-tolerant genotypes that grow vigorously at high temperatures may be more weakened by nematode infection than heat-sensitive genotypes (6). However, in testing heat-tolerant genotypes for nematode resistance, it would be favorable to correlate the temperature needed to establish some degree of resistance to *Meloidogyne.* It has long been known that the *Mi* gene for resistance is not operative in high temperatures (6,8,11).

In vitro culturing of plant-parasitic nematodes on plants produced through in vitro propagation (7,10), callus tissue, and excised root explants has been successful (2,9,15). This study was undertaken to determine if an in vitro screening technique used with excised root explants could be used for i) assessing resistance of tomato to the root-knot nematode *Meloidogyne incog-*

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nita and ii) determining the effect of temperature stress on plant response in heat-tolerant and heat-sensitive germplasms that incorporate different levels of resistance to *M. incognita.*

Materials and Methods

Plant material: Three tomato cultivars and two germplasm lines were selected for this study based their tolerance to high temperature (1) and resistance to *M. incognita* (Table 1) (personal communication, Asian Vegetable Research and Development Center, Taiwan). The two germplasm lines used were CLN 475-BC_1 F_2-265-4-19 and CL 1131-0-0-43-8-1, referred to hereafter as "CLN" and "CL", respectively. Seeds of these germplasm lines were provided by the Asian Vegetable Research and Development Center, Taiwan. The three cultivars were 'Solar Set', 'Fresh Market', and 'Rutgers'. The first two were kindly provided by Dr. J. Scott, University of Florida; the last was obtained from Meyer Seed Company, Baltimore, MD.

Root explant cultures: Seeds from each cultivar or germplasm line were surface sterilized and transferred to sterile 1.5% water agar plates (9). Seven seeds from each cultivar or germplasm line were transferred to each of six petri plates and this procedure was repeated once. The plates were maintained for 2 days in an incubator at 28 C. A 5-mm-long root tip was excised from each seedling and transferred to a sterile petri plate (two root tips per plate) containing Gamborg's B-5 medium (Gibco, Grand Island, NY). The root explants from each seedling were grown for 30 days, either without inoculation to determine adaptability to culture conditions, or inoculated with 5 egg masses per explant from cultures of *M. incognita* race 3 maintained on sterile root explants of Rutgers tomato. To determine the growth of uninoculated, excised roots, the plates were placed in a microwave oven to melt the agar, the roots were removed and weighed, and the roots were oven dried at 50 C for 24 hours and reweighed. To determine nematode penetration, root explants were removed from the plates and stained as modified from Bridge et al. (4) with cotton blue instead of acid fuschin and lactic acid-glycerol. Identification and enumeration of nematode life stages was made on a stereomicroscope and included the number of galls, second-stage juveniles (J2), third- and fourth-stage juveniles (J3 and J4), adults, and egg masses per root system. The J2 were counted as such if still vermiform or only slightly swollen, and J3 and J4 were counted as such if swollen.

Table 1. Tomato genotypes and their properties regarding tolerance to high-temperature stress and resistance to *Meloidogyne incognita.*

Genotype	Tolerance to high-temperature stress	Resistance to *Meloidogyne incognita*
CLN	Highly tolerant	Resistant
Solar Set	Moderately tolerant	Susceptible
CL	Highly tolerant	Susceptible
Fresh Market	Moderately tolerant	Susceptible
Rutgers	Intolerant	Susceptible

Plant propagation from excised roots: Plants from which the root tips were excised were maintained on the same plates under sterile conditions for seven days in an incubator at 28 C. When new roots were visible from the point of excision, the entire seedling was removed from the agar. Two seedlings each were planted into 4-cm-d Cone-Tainers (Ray Leach "Cone-Tainer" Nursery, Canby, OR) containing sterile vermiculite and placed in a growth chamber for 2 additional weeks at 28 C with a daily light cycle of 16 hours.

Seedlings were then transferred into 20-cm-d pots containing sterile soil. Each pot, containing two plants, was placed in the greenhouse (25–33 C). After 60 days, the soil was washed from the root system and the fresh weight of the whole plant was determined. Controls were run simultaneously in the greenhouse by directly sowing the seed of the test plants into 20-cm-d pots containing sterile soil.

Effect of high temperature stress on the development of nematodes: Three inoculated and 3 uninoculated root explant cultures of

each cultivar and germplasm line were placed in each of five incubators set at 28 C, 30 C, 33 C, 37 C, and 40 C, respectively. Ten days after inoculation, the number of galls in each plate was determined and the plates were then returned to their respective incubators. Roots were extracted from the agar 35 days after inoculation and stained with the cotton blue procedure. The experiment was repeated once.

Data analysis: Nematode counts were transformed using $\log_{10}$ (x + 1) before analysis. All data were subjected to an analysis of variance, and means were compared using Duncan's multiple-range test ($P \leq 0.05$) (19).

Results

Germination: Seed germination of all lines and cultivars was high (CL—100%, CLN—100%, Rutgers—98%, Fresh Market—94%, and Solar Set—91%), as was survival of seedlings after the root tips were excised. Likewise, growth of root explants at 28 C, expressed as fresh and dry weight, was comparable in all cultivars and lines (Table 2) and followed a linear growth pattern over the 30-day testing period (data not shown). The average fresh weight of a root explant at excision was approximately 95 mg and after 30 days was approximately 980 mg.

Nematode development: Both CLN and Solar Set were completely resistant to *M. incognita* at 28 C and 30 C (Table 3); and no penetration of J2 (Table 4) was detected. The only differences observed in developmental stages was that Fresh Market produced more egg masses at 28 C than the other two susceptible cultivars (Table 4).

The effect of high temperature on the development of *M. incognita* was established using J2, J3, J4, adult stages, and egg production as criteria for development (Table 4). At 28 C and 30 C, no juveniles, adults or eggs were found in the roots of nematode-resistant CLN and Solar Set. However, at 33–40 C, all stages of nematode development were detected with a low frequency at 33 C and a higher frequency at 40 C. In contrast, nematode-susceptible CL, Fresh Market and Rutgers contained many juveniles, adults and egg masses at all temperatures. Penetration occurred at 28 C, reached a maximum at 30 C, and progressively declined as temperature increased further. Although nematodes developed in both resistant and susceptible tomatoes at 37 C and 40 C, resistant CLN and Solar Set had fewer developing nematodes than the susceptible tomatoes at those temperatures.

Gall formation at high temperature: At high temperatures, galls developed on the excised roots of three tomatoes (Table 3). Fresh Market and CL were not significantly different in gall development when compared to Rutgers. High temperature

Table 2. Effect of temperature on the fresh weight of inoculated and uninoculated tomato root explants grown for 30 days *in vitro*.

Cultivar	Treatment	Fresh weight (mg)†				
		28 C	30 C	33 C	37 C	40 C
CLN	Uninoculated	1,240 c	1,032 b	823 c	735 c	781 c
	Inoculated	1,370 a	1,044 b	1,001 c	554 d	455 e
Solar Set	Uninoculated	1,065 a	590 b	1,038 a	663 c	455 e
	Inoculated	1,040 a	1,008 b	633 c	406 d	270 e
CL	Uninoculated	1,465 a	1,009 b	917 c	715 d	692 e
	Inoculated	1,460 a	1,180 b	998 b	352 c	302 c
Fresh Market	Uninoculated	1,180 a	813 b	702 c	582 d	521 d
	Inoculated	1,105 a	906 b	608 c	366 d	170 e
Rutgers	Uninoculated	1,350 a	1,147 a	442 b	207 c	182 d
	Inoculated	1,115 a	926 a	492 b	130 c	123 c

† Data are the combined results of two experiments. Means in rows followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test.

TABLE 3. Gall development on tomato root explants inoculated with egg masses of *Meloidogyne incognita* and grown for 10 and 35 days at different temperatures.

Days	Genotype	Number of galls per plate†				
		28 C	30 C	33 C	37 C	40 C
10	CLN	0 c(c)	0 c(c)	0.3 c(c)	2.3 b(a)	1.3 c(b)
	Solar Set	0 c(c)	0 c(c)	0.7 c(c)	1.6 c(b)	2.4 b(a)
	CL	3.2 b(b)	4.5 b(a)	3.2 b(b)	3.5 ab(b)	1.0 c(c)
	Fresh Market	4.3 a(b)	5.8 a(a)	6.0 a(a)	2.8 b(c)	2.7 ab(c)
	Rutgers	4.7 a(ab)	5.7 a(a)	5.3 a(a)	4.3 a(ab)	3.5 a(b)
35	CLN	0 b(c)	0 c(c)	0.8 c(b)	1.0 d(b)	2.5 ab(a)
	Solar Set	0 b(c)	0 c(c)	0.5 c(b)	2.0 c(a)	2.4 ab(a)
	CL	3.8 a(a)	4.3 b(a)	3.8 b(a)	3.2 b(ab)	1.3 b(b)
	Fresh Market	3.1 a(bc)	8.5 a(a)	8.7 a(a)	4.2 a(b)	2.5 ab(c)
	Rutgers	3.3 a(b)	6.0 a(a)	4.7 b(ab)	4.8 a(ab)	3.0 a(b)

† Analysis of the 10-day and 35-day scores were done separately. Data are the combined results of two experiments. Means in columns followed by the same letter are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test of transformed data. Means in rows followed by the same letter between parentheses are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test as transformed data.

incrementally reduced fresh weight (Table 3). The effect of high temperature and *M. incognita* on growth of root explants was determined by using final fresh weight comparisons. Greatest reduction in fresh weight was in the heat-sensitive cultivar Rutgers (87%), and lowest reduction was observed in the heat-tolerant lines CLN (37%) and CL (13%). Inoculating with *M. incognita* under high-temperature stress further reduced growth of root explants. Differences between inoculated and uninoculated treatments were lowest in nematode-resistant CLN (16%) and Solar Set (74%) and highest in heat-sensitive, nematode-susceptible Rutgers (Table 2).

TABLE 4. Effect of temperature on the development of *Meloidogyne incognita* in nematode-resistant or heat-tolerant tomatoes.

Nematode stage	Genotype	Number of nematodes†				
		28 C	30 C	33 C	37 C	40 C
J2	CLN	0 d(c)	0 d(c)	0.3 c(c)	4.3 a(a)	2.7 b(b)
	Solar Set	0 d(c)	0 d(c)	0.5 c(b)	0.5 c(b)	3.6 a(a)
	CL	5.2 b(a)	5.3 bc(a)	5.3 b(a)	3.3 ab(b)	2.3 b(bc)
	Fresh Market	7.7 a(a)	6.7 b(b)	5.0 b(c)	3.8 ab(d)	2.3 b(e)
	Rutgers	8.2 a(b)	11.0 a(a)	8.0 a(b)	4.7 a(c)	4.0 a(c)
J3 & J4	CLN	0 d(c)	0 d(c)	0.2 d(c)	3.0 ab(a)	2.0 ab(ab)
	Solar Set	0 d(c)	0 d(c)	0 d(c)	4.5 a(a)	3.1 a(b)
	CL	6.3 a(a)	6.0 b(a)	3.8 c(b)	3.8 ab(b)	1.3 c(c)
	Fresh Market	5.2 ab(a)	5.5 bc(a)	6.0 b(a)	2.0 c(b)	2.5 ab(b)
	Rutgers	6.2 a(b)	7.3 a(ab)	8.2 a(a)	2.5 c(c)	1.5 c(d)
Adults	CLN	0 d(b)	0 d(b)	0.2 b(b)	1.7 b(a)	1.3 bc(a)
	Solar Set	0 d(b)	0 d(b)	0 d(b)	1.8 b(a)	1.7 b(a)
	CL	4.3 b(ab)	5.0 b(a)	4.7 a(a)	3.2 a(a)	0.3 c(d)
	Fresh Market	7.7 a(a)	5.7 b(b)	4.7 a(c)	2.7 a(d)	2.7 a(d)
	Rutgers	7.0 a(b)	9.0 a(a)	5.2 a(c)	2.7 a(d)	1.8 b(e)
Egg Masses	CLN	0 d(b)	0 d(b)	0.7 d(a)	1.2 b(a)	1.0 a(a)
	Solar Set	0 d(b)	0 d(b)	0.7 c(a)	0.7 b(a)	0.8 b(a)
	CL	3.0 c(b)	5.0 b(a)	3.7 a(b)	3.0 a(b)	1.3 a(c)
	Fresh Market	8.7 a(a)	7.2 a(b)	3.2 a(c)	3.2 a(c)	1.3 a(d)
	Rutgers	5.7 b(b)	7.5 a(a)	3.8 a(c)	1.8 b(d)	0.8 b(e)

Data are the combined results of two experiments. Means in columns followed by the same letter and means in rows followed by the same letter in parentheses are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test with transformed data.

At day 10, nematode-resistant CLN and Solar Set, had no galls on root explants maintained at 28 or 30 C; however, galls were present on roots grown at 33 C and increased in numbers at 37 C and 40 C. In contrast, at day 10, galls appeared in large numbers on root explants of CL, Fresh Market, and Rutgers at 28 C and 30 C and, at these temperatures, in higher numbers per plate than in nematode-resistant CLN and Solar Set. The number of galls observed after 35 days was similar to the number at 10 days. However, both Fresh Market and Rutgers had more galls at 35 days on roots incubated at 30 C than at 28 C.

Discussion

The in vitro root explant technique for testing genotype resistance to nematodes offers several advantages. It is simple and requires less space, time, and labor than methods required for testing intact plants under growth chamber or greenhouse conditions. Furthermore, it can be adapted for routine screening of the large numbers of samples generally encountered in breeding programs.

The root explant test for *M. incognita* resistance should be performed between 28 C and 30 C because: i) the growth of the root explants on Gamborg's B-5 medium at these temperatures is rapid and linear during the whole 30-day testing period and ii) differences in the development of the nematode in resistant and susceptible genotypes are greatest between these temperatures, making it easier to determine their developmental stages as criteria for resistance.

Other reports (3,13) favor the use of the developmental stages, particularly egg masses because the number of eggs is a direct measure of nematode reproduction and hence, host suitability. However, we have shown that gall development is comparable to nematode development in root explants as an indicator of susceptibility.

The formation of galls and the development of juveniles, adults, and egg masses on nematode-resistant CLN and Solar Set at extremely high temperatures confirms earlier findings (11) that a reversal of host plant resistance occurs at high temperatures. Because the resistance to *M. incognita* in CLN and Solar Set results from the incorporation of the *Mi* gene into these genotypes, it seems likely that breakdown of resistance was a result of *Mi* becoming nonfunctional at high temperatures, as reported by others (11). Of course, this interpretation does not rule out the effect of metabolic dysfunctions induced by heat stress on plant vigor. These dysfunctions weaken the plant and make it more vulnerable to nematode infection (12,14,20,23).

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