The Hypersensitivity Reaction of Tomatoes Resistant to *Meloidogyne incognita*: Reversal by Cytokinins[1]

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Abstract: Initiation of larval growth, induction of cell necrosis, and gall formation in the host were measured as criteria of resistance or susceptibility of tomato seedlings to the root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood. Seedlings grown at 27 C on water agar containing additions were scored 3 or 4 days after infection.

In the absence of exogenous plant growth regulatory substances, approximately 73% of larvae that entered roots of susceptible plants showed growth, none induced necrosis and nearly all induced gall formation. In roots of a resistant variety, only 4% of the larvae grew, 88% induced necrosis of host cells, and only 29% induced galls. Exogenously supplied cytokinins shifted the response of the resistant plants toward the susceptible reaction. Exogenous kinetin at 0.4 and 0.8 μM allowed 55 and 57% of the nematodes to grow, reduced the incidence of necrosis to 32 and 31%, and increased gall formation to 73 and 65%. Three additional cytokinins, Zeatin, 6-(γ,γ-dimethylallylamino)purine, and 6-benzylaminopurine produced effects similar to kinetin. Exogenous indoleacetic acid, gibberellic acid, adenine, guanine, uracil, thymine, cytidine, and 6-methylaminopurine neither increased the percentage of larvae which grew nor decreased the extent of host cell necrosis.

Certain phytoparasitic nematodes induce pronounced alterations in the growth of their hosts, such as stunting and galling of alfalfa shoots by *Ditylenchus dipsaci* (Kühn) Filipjev, or the galling of tomato roots by *Meloidogyne* sp. Goeldi. Larvae of *Meloidogyne* enter a host at or near the root tip and migrate intercellularly to the region of differentiating vascular tissue. They become sedentary and, within a susceptible plant, the larvae begin to grow while the surrounding root tissues undergo redifferentiation. Root cells near a nematode's head display hypertrophy with repeated nuclear divisions, together with incorporation of neighboring cells; this results in large, thick-walled, multinucleate syncytia ("giant cells") (1, 2). Pericycle cells divide and enlarge to form galls; xylary differentiation is disrupted. Lateral roots frequently grow from the galls. In resistant plants, however, this pattern does not develop. Larvae may enter roots in low numbers, or the syncytia may develop abnormally. In the most common resistant reaction, larvae enter the roots, but the cells immediately surrounding the larvae die, entombing them. The nematodes presumably starve (7).

There is some evidence that the endogenous levels of plant growth regulatory substances may change during infection with *M. incognita* (Kofoid & White) Chitwood and other nematodes, but our knowledge of these changes is still fragmentary (1, 11, 12, 15, 19). In addition, exogenous plant growth regulatory substances influence the host-parasite interactions in several fungal (4, 5) and nematode (13, 20, 21) infections of plants.

The present study measured the effect of exogenous growth regulatory substances on the early stages of the host-parasite interaction between *M. incognita* and tomato seedlings. In particular, modification of the ex-

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pression of genetic resistance by cytokinins was examined.

Materials and Methods

To furnish infective larvae, tomato plants (*Lycopersicon esculentum* Mill. var. Tiny Tim) and tobacco plants (*Nicotiana tabacum* L. var. Bottom Special) were grown in a growth chamber individually in 7.6-l crocks filled with gravel. A small pump controlled by a time clock circulated Hoagland's solution to the crocks through a distributor head and plastic tubing. The solution then drained to a sump and was replaced at weekly intervals. Air temperatures were 24 C for 14 hr of light and 21 C for 10 hr of darkness. Both tomato and tobacco plants grew vigorously. The gravel in the crocks was inoculated with aseptic tomato roots infected with *M. incognita*, or with washed, chopped galls from greenhouse infections of *M. incognita*. Larvae were collected by draining fluid from the crocks through a filter consisting of two layers of 15-cm filter paper (Reeve Angel 202) in a stainless steel filter holder. The filter paper was subsequently removed and placed on a sieve in a shallow dish of water. The larvae, which migrated through the filter paper into the water, were concentrated by centrifugation.

Larvae appeared in the nutrient solution 5 to 6 weeks after inoculation and were collected twice each week. Maximum yields per collection of nematodes from 10 plants each of tobacco and tomato were 200,000 and 30,000, respectively. By the second generation of nematodes (approximately 10 weeks after inoculation), leaves were chlorotic and top growth was poor whereupon nematode numbers declined rapidly. By periodic replacement of plants, a continuous and ample supply of infective larvae was maintained for 8 months.

The three tomato varieties used in the experiments represented resistant (Y-91),[3] moderately resistant (Nemared) and susceptible (Enterpriser) hosts of *M. incognita*. Y-91 and Nemared contain the gene "Mi," and Enterpriser does not (8).

Seeds were soaked overnight in running tap water, shaken with 2% NaOCl for 6 min, rinsed with sterile distilled water and then distributed on the surface of sterile 2% water-agar in petri dishes. When roots were 1 to 2 cm long, seedlings were transferred to 1.5% water-agar containing the substances to be tested. After 24 hr, 10–20 larvae were added in a small drop of water along each root. Larvae were not aseptic.

After 3 or 4 days at 27 C roots were processed according to the following schedule: (i) fixation and staining for 4 hr in equal parts of glacial acetic acid and 95% ethanol containing 0.0175 mg/ml acid fuchsin; (ii) clearing for 12 to 24 hr in saturated aqueous chloral hydrate; (iii) final preservation and examination in clear lacto-phenol solution (17).

Infection sites were scored for larval growth, host cell necrosis and presence of a gall. Larval growth was considered "positive" when 2 of the following 3 criteria were met: enlargement of the esophageal glands, increase in body diameter, and disappearance of clear areas in the intestine (indicating an end to starvation) (18). In the great majority of cases, larval growth involved a marked increase of body diameter. Necrosis was recorded when several deformed and brown cells appeared close to the nematode's head. If a gall was associated with the larvae, galling was positive. Data are expressed as the percentage of larvae showing the above characteristics in relation

[3] Sources of seed were: Y-91, [resistant] (A. L. Harrison, Texas Agr. & Exp. Sta., Yoakum); Nemared, [intermediate] (H. B. Cordner, Oklahoma Agr. Exp. Sta., Stillwater); and Enterpriser, [susceptible] (R. E. Webb, U. S. Dept. Agr., Beltsville, Md.).

to the total number of larvae observed within the root.

6-Benzylaminopurine (BAP)[4] and 6-methylaminopurine were prepared by the general method of reacting the appropriate amine with 6-chloropurine (3, 10). Zeatin was prepared by the method of Shaw, Smallwood, and Wilson (16), and 6-(γ,γ-dimethylallylamino)purine (2iP) was prepared by the method of Hecht, Helgeson, and Fujii (9). Correct microanalyses were obtained for all compounds prepared. All other reagents were of the best commercial grade available.

RESULTS

Table 1 summarizes records of *M. incognita* growth and host response in seedlings of 3 tomato varieties. Most of the larvae (73%) which penetrated the susceptible host (Enterpriser) grew; few larvae (4%) grew in roots of the resistant host (Y-91). Failure of larvae to grow within roots was usually, but not invariably, associated with necrosis of several cells close to the nematode's head. This necrotic reaction appeared as early as 12 hr after inoculation. Necrosis of host cells was most frequent in the resistant, and never observed in the susceptible seedlings. In the susceptible and intermediate (Nemared) hosts, most larvae were located in galls (87 and 74%, respectively), whereas in the resistant host, fewer larvae (29%) appeared within galls.

TABLE 1. Percentage growth of *M. incognita* larvae and host responses in seedlings of three tomato varieties. Each seedling on water-agar was inoculated with 10 to 20 larvae and incubated for 3 to 4 days at 27 C.

Variety	Larvae within roots		
		% associated with	
	% growing	Host necrosis	Host galls
Y-91 (resistant)	4 (1145)[a]	88 (418)	29 (252)
Nemared (intermediate)	52 (721)	25 (204)	74 (204)
Enterpriser (susceptible)	73 (123)	0 (123)	87 (123)

[a] Numbers in ( ) indicate total number of larvae scored for this criterion.

TABLE 2. Influence of cytokinins on larval growth, host necrosis and host gall formation in resistant tomato seedlings. Each seedling was incubated for 24 hr at 27 C on water-agar containing the cytokinin, then inoculated with 10 to 20 larvae and incubated an additional 3 days at 27 C.

Cytokinin μM	Larvae observed within roots		Host reaction to larvae	
	Total larvae	% growth	%[a] necrosis	%[a] galls
Experiment I				
0	159	6	84	31
2iP 0.02	74	11	78	34
0.1	63	8	86	22
0.5	157	43	38	55
2.5	97	52	22	42
12.5	67	37	9	63
Experiment II				
0	65	15	79	25
BAP 2.5	139	61	29	69
Zeatin 2.5	181	46	38	74

[a] Expressed as the percentage of larvae within roots which were associated with necrosis or galls.

The proportion of nematodes exhibiting growth increased markedly in resistant seedlings on agar containing cytokinins (Table 2, Fig. 1). Furthermore, there was a great decrease in the proportion of larvae associated with necrosis of host cells and a substantial increase in larvae located within galls. A similar trend was observed with the intermediate variety, but the phenomena were less pronounced, perhaps because of the higher frequency of the susceptible-type reaction in the absence of cytokinins. No changes were noted in the susceptible variety, presumably because there was either no effect on this variety, or there was only a very small effect which our techniques could not detect. Since cytokinins produced the greatest response on Y-91, all further experiments with cytokinins were made with this variety.

[4] Abbreviations used in this paper are: 2iP, 6-(γ,γ-dimethylallylamino)purine; BAP, 6-benzylaminopurine; IAA, indoleacetic acid; GA_3, gibberellic acid.

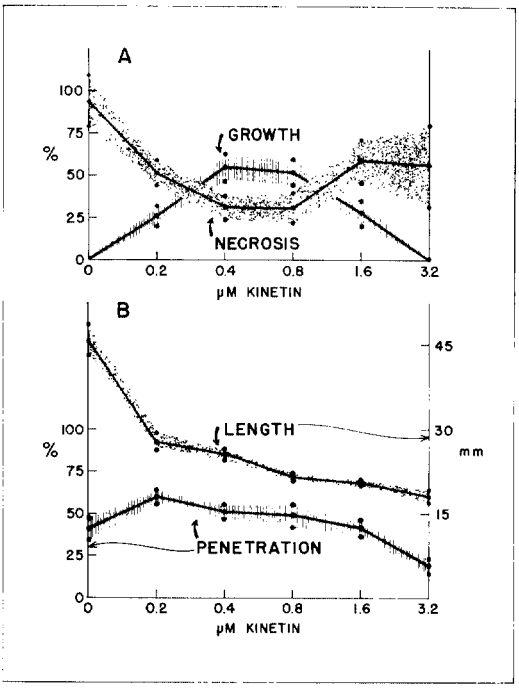

FIG. 1. Kinetin effect on (A) larval growth and host cell necrosis, and on (B) root length and larval penetration of roots. Twenty Y-91 tomato seedlings were incubated at 27 C for 24 hr on water-agar containing the appropriate kinetin concentrations. Fifteen larvae were added to each root, and the seedlings held at 27 C for an additional 4 days. Ordinate for Growth (A) = (larvae showing growth/larvae within roots) × 100; Necrosis (A) = (larvae associated with necrosis/larvae within root) × 100; Length (B) = root length in mm at end of experiment; Penetration (B) = (larvae within roots/larvae added) × 100. Curves show means and shaded areas represent 2× standard error of the mean.

Table 2 summarizes data from two experiments in which larval growth, necrosis of host cells, and gall formation were recorded for roots growing on water-agar containing cytokinins. Increased larval growth and decreased cell necrosis occurred in concentrations of 2iP as low as 0.5 μM. Zeatin and BAP had comparable effects when supplied at 2.5 μM. In all cases where these cytokinin effects on larval growth and necrosis were observed, the number of larvae associated with galls was greater than in the untreated controls.

Results from a more detailed experiment with kinetin are shown in Figure 1. In this experiment the larval age and number of larvae applied to each seedling were precisely known. Larvae were collected from 100 egg masses hand-picked from infected roots. The eggs were placed in a 1–2 mm layer of tap water and permitted to hatch for 24 hr. Fifteen larvae were added to each of 20 seedlings. Larval growth in plants grown on agar containing 0.2, 0.4, 0.8, and 1.6 μM kinetin was significantly higher, and necrosis of host cells clearly lower than controls. Thus at 0.4 to 0.8 μM kinetin the percentage of necrosis had decreased to 32 and 31%, respectively, from 97% in the control roots while the number of larvae which grew had increased from less than 1% to 55 and 52%, respectively. The maximum change from controls was observed at these concentrations. At 1.6 μM kinetin, there was less growth (28%) and more necrosis (59%). Both of these latter values were quite similar to the results obtained when some growth (to 26%) and reduction of necrosis (to 51%) was achieved by treatment with 0.2 μM kinetin. At 3.2 μM kinetin no larvae grew, although there was less necrosis than in the water control.

All concentrations of kinetin (0.2 to 3.2 μM) depressed root elongation severely but larval penetration did not change appreciably between 0.2 and 0.6 μM kinetin (Fig. 1B). At the highest concentration (3.2 μM), larval penetration decreased significantly. Those larvae which did penetrate did not grow. There was no correlation between the length of individual roots 4 days after inoculation and the degree to which the larvae penetrated. Furthermore, there was no apparent difference among the susceptible,

intermediate, and resistant varieties with regard to cytokinin effects on root length.

The minimum concentration of 2iP necessary to elicit the larval growth response was the same as that for kinetin, 0.2 μM. However, 2.5 μM 2iP gave a greater increase in larval growth and reduction of necrosis than 0.5 μM 2iP (Table 2). Even 12.5 μM 2iP promoted larval growth, although to a lesser extent than 2.5 μM 2iP. Thus, kinetin promotes larval growth only within the range 0.2 to 1.6 μM, and is inactive at 3.2 μM, but 2iP is active over the range 0.2–12.5 μM. This is more than 4 times the effective concentration range of kinetin. Moreover, this is a lower limit, since higher concentrations of 2iP were not tried. This broader range of maximum activity for 2iP is similar to that observed in other systems, e.g., the tobacco callus total growth bioassay (14).

By a similar comparison with kinetin we found the lower limit of zeatin activity was slightly higher than for kinetin. The percentage of larvae which grew was not increased by 0.1 μM zeatin, may have been affected by 0.2 μM and was definitely increased by 0.4 μM zeatin. The possibility that high concentrations of zeatin may be inhibitory (c.f. 3.2 μM kinetin in Fig. 1) was not examined. However, 2.5 μM zeatin was highly active in increasing the number of larvae which grew, and in decreasing the necrotic response, as was the same concentration of 2iP (Table 2).

In addition to promoting initial development of the nematode infection, applications of cytokinins allowed the completion of the life cycle. Resistant seedlings were grown aseptically on modified White's medium. Thirty-six days after an aseptic egg mass of *M. incognita* was added to each tube, 11 plants grown on the medium without kinetin had 3 galls, including 1 small egg mass, and 9 plants grown on medium containing 0.5 μM kinetin had 8 galls including 4 moderate-sized egg masses.

In the experiments discussed above, cytokinins, where added, were present for the duration of the experiment. To test whether applications of cytokinins to the nematode alone affect the host-parasite interaction, the following experiment was performed. Larvae were incubated for 24 hr in water or aqueous solutions of kinetin (0.5, 1.0, or 2.0 μM). The larvae were then washed thoroughly with water and placed on resistant seedlings growing on agar which did not contain exogenous cytokinins. A total of 50 larvae were scored for each treatment. There were no observable differences in penetration, induction of necrosis, or larval growth between larvae incubated in water and those in kinetin solutions. These results indicate that the cytokinin effect was not achieved by treating the nematodes prior to their contact with resistant seedlings.

Of the compounds tested only cytokinins known to be highly active at low concentrations in other test systems increased the susceptibility of resistant tomato seedlings to *M. incognita* infection. Adenine and guanine were tested separately at 0.5, 2.5, and 12.5 μM. Uracil, thymine, and cytidine were tested together in equimolar solutions at the above concentrations for each substance. 6-Methylaminopurine was tested at 100 μM. None of these treatments had a significant effect on nematode growth, whereas the 2iP control (0.5 μM) increased larval growth more than 3-fold. Exogenously supplied IAA (0.29 to 1.14 μM) or GA_3 (2.9 to 290 μM) failed to stimulate growth of larvae in seedling roots.

DISCUSSION

The interaction between tomato plants and the invading nematode, *M. incognita*, results in rather drastic changes in both organisms. Within a susceptible host (var.

Enterpriser) the nematode exhibits growth, differentiation, change in growth habits, and ultimately, reproduction. The susceptible root forms giant cells and galls, with the accompanying hypertrophy and hyperplasia. When the nematode invades a resistant (var. Y-91) plant, another change (that of a rapid necrosis of tissue surrounding the nematode) occurs. In the presence of the appropriate cytokinin concentrations, this resistant interaction was altered and the plants became more susceptible to *M. incognita* infections. Other plant growth regulatory substances (IAA, GA_3) had no effect at the concentrations tested. Similarly, other purines or pyrimidines, either without cytokinin activity (guanine, thymine, cytidine, uracil), or with weak cytokinin activity (6-methylaminopurine and adenine) failed to modify the resistance reaction.

The induction of galls is not completely dependent on either larval growth or lack of necrosis of host cells. The proportion of larvae in galls was consistently higher in all three tomato varieties than the proportion of grown larvae (Table 1). Further, in the untreated resistant seedlings of the 2iP series of Table 2, 6% of the larvae in the roots grew and did not incite necrosis; an additional 10% neither induced necrosis nor grew; the remaining 84% were associated with host cell necrosis. Since 31% of the larvae were in galls, at least 15% of the larvae which were associated with host cell necrosis also induced galls.

Necrosis of host cells and larval growth are mutually exclusive. Necrosis diminished in all treatments in which larval growth was higher than in the control; but in some preliminary experiments, it also diminished in seedlings treated with 6-benzyl adenine and zeatin at levels below those which stimulated larval growth. High cytokinin concentrations also produced irregular results. No larvae grew at 3.2 μM kinetin (Fig. 1), yet only 56% of the larvae within the roots were associated with necrosis, compared with 97% necrosis in the roots without exogenous cytokinin. In another experiment (Table 2), both growth (37% vs. 52%) and necrosis (9% vs. 22%) were lower at 12.5 μM than at 2.5 μM 2iP. These results indicate that a decrease in necrosis *can* occur in the absence of a concomitant increase in larval growth.

There is little evidence bearing on the mechanism of the cytokinin effect on the resistant → susceptible transition. Since kinetin had no apparent effect when applied to the larvae *prior* to infection, the cytokinin is not acting on the larvae alone. This does not preclude an effect on the larvae once they enter the very different environment of the root. More probably, however, cytokinin alters the physiological state of the root. There is no evidence that a nematocidal toxin is produced in resistant tomatoes. Larvae removed by dissection from such plants in the present experiments several days after penetration were still infective to susceptible tomato seedlings.

We conclude that the nematode and the root must exchange some kind of "signal" very soon after the larva has entered the root. Since we have observed necrosis beginning as early as 12 hr after infection, and since galling has been observed as early as 24 hr after infection(s), the signal(s) which elicited these responses probably acted soon after penetration. However, the nature of the signal(s) is completely unknown.

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