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Influence of Metyrapone on Development of Heterodera glycines[1]

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Abstract: Metyrapone, an inhibitor of steroid synthesis, affected the survival and rate of development of *Heterodera glycines*. Metyrapone in aqueous tartaric acid solvent influenced sex ratios. The effect on sex ratios was mediated through the host, whereas the effect on survival was apparently effected directly. Tartaric acid increased larval penetration of soybean roots. *Key Words:* sex ratio, cyst nematodes, soybean.

Several workers (2, 5, 8) have suggested that the diverse environmental factors known to influence the sex ratios of nematodes may have a common biochemical mechanism that is hormonally mediated. Fox *et al.* (2) hypothesized that host-plant steroids may: 1) influence the nematode's metabolism of growth and molting hormones to produce a type of resistance which reduces the number of nematodes; or 2) influence the metabolism of the nematode's sex hormones to produce a type of resistance which induces most larvae to become males. Recent research (2, 5, 7) indicates that exogenous sex hormones may affect nematode growth and development, although sex expression has not been influenced. The selection of exogenous hormones for experimentation is currently an empirical procedure since it is not well understood which hormones influence sex expression in nematodes, or how exogenous hormones may interact with endogenous hormones.

Inhibitors of hormone synthesis represent a possible means of manipulating endogenous hormones. Since estrone influenced the susceptibility of soybean to *Heterodera glycines* (2), metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), an inhibitor of estrone synthesis in mammals (4), was chosen for investigation. It was assumed that metyrapone could have a mode of action in the nematode-host-plant system similar to that reported for mammals.

The study was done to determine whether metyrapone, a known inhibitor of estrone synthesis, could influence the development and sex differentiation of *Heterodera glycines*.

MATERIALS AND METHODS

The soybean variety 'Lee' (*Glycine max*), susceptible to *H. glycines*, was selected as the host plant for all the experiments. A line of soybean was increased from a single seed to minimize experimental error due to genetic variability of the host. Seedlings 7 days old, germinated in a 1:1 v/v mixture of Weblite (Weblite Corporation, Roanoke, Virginia) and vermiculite, were carefully washed free of the planting mixture and inoculated with second-stage larvae.

In inoculations, an aliquot of water containing larvae of *H. glycines*, isolate Arkansas 1, was transferred by pipette to a 25 × 100-mm plastic petri-dish bottom and tap water was added to cover the dish bottom and disperse the larvae. An individual seedling was transferred to the dish, and coarse white sand was added to cover the soybean roots and fill the petri-dish bottom. The plants were watered with tap water, covered with a plastic petri-dish top, and placed on a support bench, where they were maintained to allow the larvae to penetrate the seedling roots.

After the inoculation period, the sand was gently rinsed from the soybean roots and the plants were transferred to a hydro-

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ponic system containing either Hoagland's nutrient solution (6) or metyrapone solutions. The containers used to hold the hydroponic solutions were modified 1-liter plastic containers. A circular 2.5-cm hole was cut out of the four corners of each container lid to allow the soybean shoot to protrude, and a glass tube was inserted to aerate the solutions. Air under pressure was supplied by a 1.3-amp Neptune Dyna Pump (Universal Electric Co., Owosso, Michigan). The plants received continuous lighting under Westinghouse cool white fluorescent lamps (500 lux), and temperature was maintained at 23 to 28 C.

The treatment solutions used in the metyrapone experiments were as follows: 1) water check, tap water only; 2) solvent check, an aqueous solution of tartaric acid at 150 μg/ml; 3) a solution of Metopirone (Ciba-Geigy, Summit, New Jersey), a commercial preparation of metyrapone, at 4 μg/ml and tartaric acid at 6 μg/ml; 4) a solution of Metopirone at 20 μg/ml and tartaric acid at 30 μg/ml; and 5) a solution of Metopirone at 100 μg/ml and tartaric acid at 150 μg/ml. The pH of each solution was adjusted to 6.5 with 1% or 10% potassium hydroxide.

Recovery of nematodes for determination of sex ratio was in three phases. Nineteen and 26 days after inoculation the hydroponic solutions were sieved through a 26-μm screen to recover males. Twenty-six days after inoculation the host roots were washed forcefully with running water to dislodge females from the roots, and the females were collected on a 26-μm screen. After the roots were rinsed, they were fixed and stained in a 1:1 v/v solution of glacial acetic acid and absolute ethanol containing acid fuchsin and cleared with a saturated chloral hydrate solution to reveal nematodes embedded in the roots.

The influence of metyrapone on the sex differentiation of *H. glycines* was determined in an experiment in which the roots of soybean seedlings were simultaneously inoculated with nematode larvae and treated with metyrapone (Expt. A). Individual seedlings were transferred to modified petri dishes, where they were inoculated with about 800 larvae, and an appropriate treatment solution of water, aqueous tartaric acid, or Metopirone at 4, 20, 100 μg/ml in tartaric acid was added at a sufficient volume to saturate the sand. The inoculated and treated plants were maintained for 7 days in the petri dishes and watered with treatment solutions as needed to prevent desiccation.

After the inoculation and treatment period, the roots of four plants per treatment were fixed, stained, and cleared to determine the number of nematodes in the roots. Twelve plants of each treatment were transferred to the hydroponic system containing Hoagland's nutrient solution. There were four plants per container (replicate) and three replicates per treatment. Nematodes were recovered and the sex ratio was determined.

To determine whether any influences of metyrapone on sex differentiation were the result of metabolic changes within the nematode, different concentrations of metyrapone were applied to the nematodes alone before inoculation (Expt. B). Larvae were transferred to the same five treatment solutions described above in covered 100-ml beakers and placed in the dark at room temperature (25 C) for 48 h. The beakers were agitated gently at intervals to aerate the suspensions. Individual soybean seedlings were transferred to modified petri dishes, inoculated with about 200 treated larvae and maintained in the petri dishes for 2 days.

After the inoculation period, the roots of five plants per treatment were fixed, stained, and cleared to determine the larval penetration. Twelve plants of each treatment were transferred to the hydroponic system containing Hoagland's nutrient solution. There were four plants per container (replicate) and three replicates per treatment. Nematodes were recovered and the sex ratio was determined.

To determine whether the influences of metyrapone on sex differentiation of *H. glycines* were the result of changes in the host plant metabolism, and to control the nematode population density in the host roots, metyrapone was applied to the host plant 48 h after inoculation (Expt. C). Individual seedlings were transferred to modified petri dishes, inoculated with about 200 larvae, and maintained for two days. After the inoculation period, 12 plants were fixed, stained, and cleared to determine the

larval penetration per plant. Twelve plants per treatment were transferred to the hydroponic system containing the five treatment solutions and maintained for 5 days. There were two plants/container (replicate) and six replicates/treatment. After the treatment period, the treatment solutions were replaced with Hoagland's nutrient solution, in which the plants were maintained until the nematodes were recovered and the sex ratio determined.

The percentage of survival to adulthood was calculated from the total number of adults and total number of larvae in the roots immediately after the inoculation period. The latter number was derived by multiplying the mean penetration per plant by the total number of plants per treatment.

The sex ratios obtained within a treatment were tested with chi-square for goodness of fit to a ratio of 3 males to 1 female as shown (1) to be characteristic for this isolate under these conditions. Treatment differences within an experiment were analyzed with Duncan's multiple-range test. Arc-sine transformations were used in the analysis of data expressed as percentages.

RESULTS

Metyrapone applied to soybean roots at low (4 μg/ml) and intermediate (20 μg/ml) concentrations had no apparent effect on soybean morphology. In contrast, the root system of the plants in the high metyrapone concentrations (100 μg/ml) were stunted and discolored, with sparse tertiary root proliferation, whereas the root systems of the water and solvent check plants were elongated and white, with abundant tertiary root proliferation. The primary leaves of plants in the high metyrapone treatments were stunted with irregular to circular, necrotic, tan spots with purple margins. The trifoliolate leaves of these plants did not emerge until the plants were removed from the treatment solutions, and were stunted, curled, and roughened after they emerged. The cotyledons remained green and abscission of the cotyledons was delayed. In contrast, shoots of plants in the other treatments appeared normal. The primary leaves enlarged, the trifoliolate leaves maintained a typical appearance, and the cotyledons turned yellow and withered.

When metyrapone treatments accompanied nematode inoculation (Expt. A) the percentage of males among adults was significantly lower in these treatments than in the water check, but was not lower than in the aqueous tartaric acid solvent check (Table 1). In addition to the influence on

TABLE 1. Development of *Heterodera glycines* treated with metyrapone at inoculation.

	Nematodes in roots[wy]	Percentage survival to adult	Total adults[x]	Percentage males	Chi-square test 3 males: 1 female
Water	159.0bc	27.4ab	174.3a	77.1b	1.07
Aqueous tartaric acid (150 μg/ml)	177.5c	19.0a	134.7a	68.6ab	8.59[**z]
Metyrapone (4 μg/ml) in tartaric acid (6 μg/ml)	139.5bc	34.2b	190.7a	67.1a	18.46**
Metyrapone (20 μg/ml) in tartaric acid (30 μg/ml)	118.3ab	42.0bc	198.7a	59.1a	79.91**
Metyrapone (100 μg/ml) in tartaric acid (150 μg /ml)	75.3a	50.1c	150.7a	60.6a	49.09**

[w]Mean of four plants, sampled immediately after the penetration period.
[x]Mean of three replicates with four plants per replicate.
[y]Means in the same column followed by the same letter are not significantly different by Duncan's multiple-range analysis (P = 0.05).
[z]Asterisks significantly different from that expected (P = 0.01).

sex ratio, the metyrapone treatments influenced nematode growth and development in Expt. A. Metyrapone suppressed larval penetration, increased the rate of emergence of males from the roots, and increased the percentage of survival to adulthood. Percentage of survival to adulthood increased with the concentrations of metyrapone. About 97% of the total number of males had emerged 19 days after inoculation in the high metyrapone treatments. This rate was in contrast to 34.2% in the water check and 36.5% in the aqueous tartaric acid check (Table 2).

The application of metyrapone to the nematode larvae prior to inoculation (Expt. B) did not influence the sex ratio of the developing nematodes (Table 3). Although sex ratio was not affected, growth and development were, as evidenced by the effect of metryapone on percentage survival to adulthood, which was increased significantly by the high metyrapone treatment. The aqueous tartaric acid check also increased nematode penetration.

When metyrapone was applied to the host plant 48 h after inoculation (Expt. C) there was no significant influence of metyrapone on sex ratio or percentage survival to adults, although the percentage of males was arithmetically lower in the metyrapone treatments (Table 4).

TABLE 2. Effect of metyrapone on emergence of *Heterodera glycines* males from roots of 'Lee' soybeans.

	Total[x] males	Percent emergence at 19 days
Water	403	34.2
Aqueous tartaric acid (150 μg/ml)	277	36.5
Metyrapone (4 μg/ml) in tartaric acid (6 μg/ml)	384	44.8
Metyrapone (20 μg/ml) in tartaric acid (30 μg/ml)	353	68.5
Metyrapone (100 μg/ml) in tartaric acid (150 μg/ml)	274	96.7

[x]Sum of males recovered from twelve plants per treatment at 19 and 26 days after inoculation.

DISCUSSION

Results of metyrapone treatments of *H. glycines* larvae and soybean roots during ingress (Expt. A) indicate that metyrapone or the interaction of metyrapone and the aqueous tartaric acid could shift the sex ratio of *H. glycines* while increasing the survival rate of the nematode. For the shift

TABLE 3. Development of *Heterodera glycines* treated with metyrapone before inoculation on soybean roots.

	Nematodes in roots[w y]	Survival %	Total adults[x]	Percentage males	Chi-square test 3 males: 1 female
Water	32.4b	79.6b	103.0c	79.0a	2.38
Aqueous tartaric acid (150 μg/ml)	104.2a	75.2b	313.3a	73.9a	0.57
Metyrapone (4 μg/ml) in tartaric acid (6 μg/ml)	54.2b	74.4b	161.3b	70.7a	4.63*[z]
Metyrapone (100 μg/ml) in tartaric acid (150 μg/ml)	49.6b	100.0a	201.3b	77.0a	1.17

[w]Mean of five plants, sampled immediately after the penetration period.
[x]Mean of three replicates, with four plants per replicate.
[y]Means in the same column followed by the same letter are not significantly different by Duncan's multiple-range analysis (P = 0.05).
[z]Asterisk, significantly different from that expected (P = 0.05).

TABLE 4. Effects of postinoculation treatment of soybean roots with metyrapone on nematode development of *Heterodera glycines*.

	Nematodes in roots[w]	Survival %[x]	Total adults[y]	Percentage males	Chi-square test 3 males: 1 female
Water	20.6	41.7a	103a	66.0a	3.97*[z]
Aqueous tartaric acid (150 μg/ml)	20.6	51.6a	85a	50.1a	25.73**
Metyrapone (4 μg/ml) in tartaric acid (6 μg/ml)	20.6	36.4a	90a	60.0a	10.02**
Metyrapone (20 μg/ml) in tartaric acid (30 μg/ml)	20.6	37.2a	92a	53.3a	22.04**
Metyrapone (100 μg/ml) in tartaric acid (150 μg/ml)	20.6	45.3a	112a	59.8a	12.96**

[w]Mean of 12 plants, sampled immediately after penetration period.
[x]Total of 12 plants per treatment.
[y]Means or totals in the same column followed by the same letter are not significantly different by Duncan's multiple-range analysis (P = 0.05).
[z]Asterisks (*, **) indicate different from those expected at P = 0.05 and 0.01, respectively.

in sex ratio caused by the chemical treatment to be due to a differential death rate, the male sex would have had an increased death rate. Since that is contrary to previous reports of differential death rates and since the metyrapone treatments increased the survival rate, this experiment indicates that the tendency of a larva to become female was increased by the metyrapone treatments. Trudgill (9) reported that DL-tyrosine increases the tendency of *Globodera rostochiensis* larvae to become male. It is not yet clear how the chemical environment of the nematode can influence its sex differentiation. However, the effect of exogenous estrone on the growth and development of *H. glycines* on soybeans as reported by Fox et al. (2) and the apparent effect of metyrapone on sex differentiation emphasizes the possible role of the steroids as nematode sex hormones.

The metyrapone treatments had no effect on sex ratios in Expt. B when the nematodes were treated before inoculation. The sex ratios in three of the four treatments were consistent with the ratio of 3 males to 1 female reported for the Arkansas 1 isolate of *H. glycines* (1). This indicates that the action of the metyrapone treatments in Expt. A may involve the host in its influence on sex differentiation. The failure of Expt. C to duplicate the results of Expt. A may indicate the first hours of feeding are critical in determining a larva's sex.

Sex differentiation of *H. glycines* was apparently influenced by the components of the metyrapone treatments acting either separately or together. This phenomenon may be similar to the conversion of genic males to reproductive females by exogenous estrogens as confirmed by genetic test crosses in *Xenopus laevis* (10). Alteration of the estrogenic balance was reported to exert a female-inducing influence in birds also (3).

The depressed larval penetration that resulted from a high concentration of metyrapone applied at the time of inoculation could be attributed primarily to suppressed root growth of the plants, with subsequently fewer penetration and feeding sites. Soaking larvae in metyrapone solutions for 48 h before inoculation (Expt. B) did not influence larval penetration, although the same exposure to the aqueous tartaric acid check significantly stimulated larval penetration, as also in Expt. A,

though to a lesser extent. Metyrapone apparently counteracted the effect of the solvent solution on penetration.

Metyrapone increased the rate of emergence of males when roots were inoculated and treated simultaneously. The percentage of total males observed 19 days after inoculation increased linearly with the concentration of metyrapone, suggesting that males developed faster in the debilitated plants (metyrapone at 100 μg/ml) than in the apparently healthy plants (water check).

Not all nematodes that entered the soybean roots survived to maturity. Percentage survival increased significantly when high levels of metyrapone were applied to the host root at the time of inoculation. Survival increased also when the nematodes alone were treated, though not when the roots were treated 48 h after inoculation. The increased survival in the high-metyrapone treatments of both the debilitated plants of Expt. A and the apparently healthy plants of Expt. B indicated that this effect was a function of metyrapone influences on the nematode's metabolism and not the physiological state of the host plant. The influences of metyrapone on nematode growth and development in the present study may suggest that steroid compounds are involved in nematode growth and development.

LITERATURE CITED

1. EVANS, D. M., and J. A. FOX. 1977. The sex ratio of Heterodera glycines at low population levels. J. Nematol. 9:207-210.
2. FOX, J. A., L. H. AUNG, and A. J. WEBBER. 1972. The relationship of a steroid to resistance of soybean to Heterodera glycines. J. Nematol. 4:224 (Abstr.).
3. GAARENSTROOM, J. H. 1939. Geschlectsbeeinflussung durch diathylstilboestrol. Acta. Brevia Neerlandica de Physiologia 9:13-14.
4. GOWER, D. B. 1974. Modifiers of steroid-hormone metabolism: a review of their chemistry, biochemistry and clinical applications. J. Steroid Biochem. 5:501-523.
5. HANSEN, E. L., and E. J. BUECHER. 1971. Effect of insect hormones on nematodes in axenic culture. Experientia 27:859-860.
6. HOAGLAND, D. R., and D. I. ARNON. 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Cir. 347: 1-32.
7. JOHNSON, R. N., and D. R. VIGLIERCHIO. 1970. Heterodera schactii responses to exogenous hormones. Exp. Parasitol. 27:301-309.
8. LAUGHLIN, C. W., A. S. WILLIAMS, and J. A. FOX. 1969. The influence of temperature on the development and sex differentiation of Meloidogyne graminis. J. Nematol. 1:212-215.
9. TRUDGILL, D. L. 1974. The influence of DL-tyrosine on the sex of Heterodera rostochiensis. Nematologica 20:34-38.
10. WITSCHI, E. 1971. Mechanisms of sexual differentiation. Pages 601-618 *in* M. Hamburgh and E. J. W. Barrington eds. Hormones in development. Appleton-Century-Crofts, New York.