Physiology of Sexual Reproduction in Hypomyces solani f. sp. cucurbitae

VI. The Effects of Tyrosinase and Cold Treatment on Cellular Permeability

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ABSTRACT

Cultures of *Hypomyces solani* f. sp. *cucurbitae*, race 1, were exposed to 4°C or were treated with tyrosinase at 10 days to study the effects of these treatments on cellular permeability and cellular tyrosine. Leakage values from cultures growing in potato-dextrose broth were similar after the cold treatment or enzyme treatment. There was less K^+ leakage, more Mg^{++} leakage, and more amino acid leakage from the treated cultures than from the control cultures. No differences were observed in the Na^+ and Ca^{++} leakages. There was a decrease in the free tyrosine 1 day after either treatment, but 5 days after either treatment, control and treated cultures were similar. Less tyrosine was found in protein hydrolysates from treated cultures than from nontreated cultures, but recovery was complete within 10 days after treatment. These results support the hypothesis that extracellular tyrosinase alters membrane permeability. This permeability change may be the initial factor in the differentiation processes involved in the stimulation of the production of perithecial primordia, with melanin production being of secondary importance. There is no evidence than this mechanism is responsible for maintaining the permeability differences observed. Phytopathology 60:129-131.

A positive correlation between tyrosinase activity and the formation of perithecial primordia in *Hypomyces solani* f. sp. *cucurbitae*, race 1 was demonstrated in a previous paper in this series (15). Tyrosinase activity or melanin formation has also been associated with morphogenesis in species of *Neurospora, Glomerella, Verticillium,* and *Blastocladiella* (3, 4, 9, 12, 14).

In addition to its role in melanin formation, tyrosinase is able to oxidize tyrosine moieties in purified proteins (6, 13, 16). In some cases, oxidation of the tyrosyl groups of biologically active proteins resulted in the loss of biological activity (6). Cory (5) reported that purified tyrosinase caused lysis of red blood cells, swelling of Ehrlich ascites cells, and permeability changes in isolated rat diaphragms. These changes were prevented by tyrosinase inhibitors in the incubation mixture. These results indicated that tyrosine may be an important structural feature of membranes, and may play a role in the control of membrane permeability.

The present investigation utilized strain 4 of *Hypomyces solani* (Mart.) Appel. & Wr. f. sp. *cucurbitae* Snyd. & Hans., race 1, in an attempt to establish (i) if any cellular permeability change occurred in the transition from vegetative to sexual growth; and (ii) if the free and protein tyrosine contents of the cells were altered during the induction of morphogenesis.

MATERIALS AND METHODS.—Cultures of *H. solani* initiated from single conidia were used to determine the Na^+, K^+, Ca^{++}, Mg^{++}, and amino acid leakage from 10-, 11-, 15-, 20-, 25-, and 30-day mycelial mats grown in 30 ml of potato-dextrose broth (PDB). The laboratory conditions, the medium, and the strain 4 clone of *H. solani* were previously described (15). The cultures which had been grown under laboratory conditions were exposed to 4°C for 4 hr at 10 days and then returned to room temperature, or received 1 ml of 0.5 mg/ml aqueous tyrosinase (Nutritional Biochemicals Corp.)/30 ml of liquid medium at 10 days. The control or treated mycelial mats were removed from the medium, washed with water, and placed in a nearly isotonic unbuffered 5.5% ascorbic acid solution for 4 hr. The mycelial mats were then removed by filtration. Some cultures were treated for 30 sec with hot acetone, then placed in the ascorbic acid solution for 4 hr. At least 5 determinations were made for each leakage value. After a 1:10 dilution with water, K^+ and Na^+ leakage values were determined, using a Model KY Baird-Atomic Flame Photometer. Mg^{++} and Ca^{++} concentrations were determined using a Perkin-Elmer Model 290 atomic absorption spectrophotometer. Total amino acid leakages were determined, using the method of Yemm & Cocking (17), with glutamic acid as the standard.

Concentrations of extractable free tyrosine and protein tyrosine obtained by hydrolysis were determined for cultures grown in PDB under laboratory conditions, exposed to 4°C, or treated with tyrosinase on the 10th day. The cultures were washed with water and placed in hot acetone. The free tyrosine was extracted by macerating the mycelial mats in 10 ml of 80% acetone. The extracts were concentrated to 2 ml and washed three times with 2 ml of ethyl ether. The extracts were then brought up to 25 ml with 80% acetone. No correction was made for tyrosine lost during extraction. The macerated residues containing precipitated protein and cell-wall material were dialyzed at 2°C for 2 days against water. The dialyzed residues were hydrolysed in 20 ml of 10N H_2SO_4 for 10 hr at 95°C. The protein hydrolysates were filtered and washed three times with

3 ml of ethyl ether. Free and protein tyrosine contents of the solutions were estimated by measuring the absorbance at 480 nm after adding freshly prepared Millon's reagent (10). Samples containing 0.02 mg to 0.15 mg tyrosine/ml were used in all cases. Paper chromatograms were run in 5% acetic acid to determine if tyrosine was the only compound in the extracts giving a positive Millon reaction. Other Millon positive spots gave a positive Arnow's test. A correction for ortho-dihydroxy benzene derivatives was made at 500 nm using Arnow's reagent (1) as described by Harrison (8), with dopa as the standard. The amount obtained from Arnow's positive reaction was subtracted from that of the Millon test, to give an estimation of tyrosine.

RESULTS.—Cultures growing in PDB were used to determine the leakage of Na^+, K^+, Ca^{++}, Mg^{++}, and amino acids from 10-, 11-, 15-, 20-, 25-, and 30-day cultures. These results, except Na^+ leakage, are summarized in Fig. 1. Results were reported as leakage into 10 ml of 5.5% ascorbic acid from mycelial mats; the values were corrected to 200 mg dry mycelium. Variation of the Na^+ leakage between sample replicates was greater than the differences between treatments, and data for this ion are not shown. The other leakage values showed little variation within replications. The leakage from the acetone-treated mycelial mats showed that Na^+ is not tightly bound inside the cells, or that it is present in low concentrations in the cells. There was less leakage of K^+ from cold-treated or enzyme-treated mycelia than from untreated mycelia. There was little difference in the Ca^{++} leakage between treated cultures and the control cultures. However, these leakage values were much lower than those from acetone-treated mycelial mats. The leakage of Mg^{++} from mycelial mats was increased by enzyme or cold treatment, but this leakage was low compared to acetone-treated mycelia. Total amino acid leakage was increased in the cold-treated or enzyme-treated mycelial mats.

Free and protein tyrosine contents were assayed at 10, 11, 15, 20, 25, and 30 days. The amounts of tyrosine extracted by acetone were very low in the 11-day cultures which had been enzyme or cold treated the previous day (Fig. 2, left). There was little difference in the amounts of free tyrosine after the 15th day. Figure 2, right, shows the amounts of protein tyrosine present following hydrolysis of the macerated, dialyzed mycelium. There was a decrease in the amount of protein tyrosine on the 11th and 15th days in the cold-treated and enzyme-treated cultures. After 20 days, there was little difference in the protein tyrosine content. However, there was more total protein at 25 and 30 days in cultures exposed to the cold or enzyme treatment than in the controls.

DISCUSSION.—The effect of tyrosinase on the permeability of the cellular membranes may be an important factor in the increased production of perithecial primordia (15). In this investigation, leakage values of inorganic ions and amino acids from intact mycelial mats were used to give a relative indication of the permeability changes of the cellular membranes in *H. solani* as a result of cold or tyrosinase treatment.

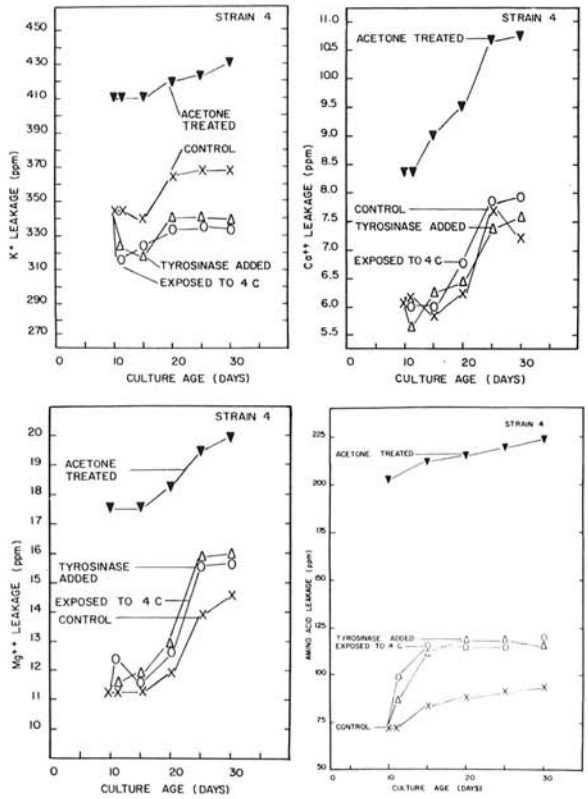

Fig. 1. K^+, Ca^{++}, Mg^{++}, and amino-acid leakage from mycelial mats of *Hypomyces solani* f. sp. *cucurbitae*, strain 4, as affected by the addition of 1 ml of 0.5 mg/ml aqueous tyrosinase or exposure to 4°C for 4 hr at 10 days. The acetone treatment represents leakage from nonviable cells. Leakage is expressed as parts per million into 10 ml of 5.5% ascorbic acid (corrected to 200 mg dry mycelium).

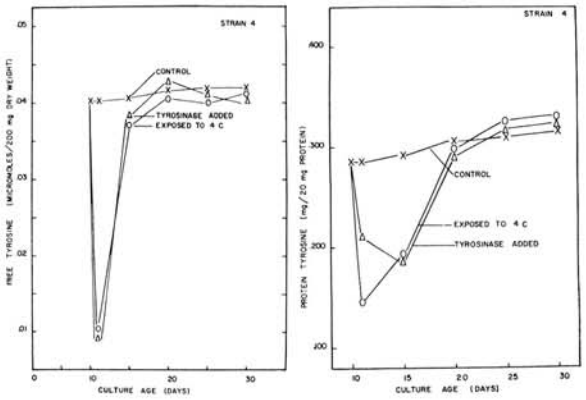

Fig. 2. (Left) Free tyrosine extracted by acetone from cultures of *Hypomyces solani* f. sp. *cucurbitae*, strain 4, grown in PDB and exposed to different conditions. Tyrosine is expressed as μmoles of tyrosine per 200 mg of dry mycelium. (Right) Effect of the addition of 1 ml of 0.5 mg/ml aqueous tyrosinase or exposure to 4°C for 4 hr at 10 days on the tyrosine content of total mycelial protein of *Hypomyces solani* f. sp. *cucurbitae*, strain 4. Tyrosine is expressed as mg of tyrosine per 20 mg of protein.

Nearly isotonic ascorbic acid was used because it does not activate tyrosinase. Leakages from cold-treated or tyrosinase-treated mycelial mats were similar in all cases. These results suggest that the same mechanisms of action are involved in both cases.

Perhaps the permeability change effected by the action of tyrosinase was highly selective. No explanation can be offered at present, using these results, for the decrease in K^+ leakage and increase in Mg^{++} and amino-acid leakage from treated cultures. Studies on active ion uptake and metabolism would be necessary before adequate explanations could be offered for the differences observed. Ca^{++} has been shown to be important in membrane integrity (2), and it is one of the least mobile inorganic electrolytes (11). Large Ca^{++} leakage values would be expected only from acetone-damaged membranes; this was observed to be the case.

Tyrosine assays were made at 5-day intervals in cultures growing in PDB. Less free tyrosine was found in the 11-day cultures exposed to the cold treatment or treated with tyrosinase than in the controls. However, there was little difference in the free tyrosine after 15 days. Darkening of the medium and the surface of the mycelial mat occurred soon after an exposure to either tyrosinase or cold treatment. Further darkening was not observed after the 11th day; thus, melanin formation from free tyrosine probably occurred only at that time.

Less tyrosine was found in the protein hydrolysates from the 11- and 15-day cultures in the cold- and enzyme-treated cultures than in control cultures. After 20 days, there was little difference in the tyrosine content of the protein hydrolysates. Although hydrolysates of total protein were used, the differences in the tyrosine content may be due to differences only in the membrane proteins. These results support the hypothesis that tyrosinase alters the permeability of cellular membranes.

Since tyrosinase activity diminishes with the age of the cultures (15) and there is no difference in the amount of free or protein tyrosine in the cultures after 20 days, there is no evidence that this mechanism is responsible for maintaining the permeability differences. Perhaps the alteration of the effective concentrations of inorganic electrolytes after the cold treatment or enzyme treatment is responsible for maintaining these differences, and for the subsequent differentiation process. Harman's (7) observation that KCl in the culture medium stimulated the production of primordia may support this idea. Perhaps changes in the effective concentrations of K^+ and Mg^{++} inside *H. solani* cells affect the chromosomal activity, and this controls the subsequent differentiation processes.

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