Effect of Ethrel and Ceratocystis fimbriata on the Accumulation of Chlorogenic acid and 6-Methoxy Mellein in Carrot Root

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ABSTRACT

The concentration of 6-methoxy mellein (MM) increased in the upper millimeter of carrot discs treated with a solution of Ethrel (2-chloroethyl phosphonic acid). The concentration during a 5-day interval after treatment was equal to or slightly greater than that in discs inoculated with *Ceratocystis fimbriata*. Little or no MM was found in fresh or aged discs. MM accummulation increased in carrots treated with increasing concentrations of Ethrel up to 9 mg Ethrel/ml. Chlorogenic acid concentration in the upper millimeter of carrot discs increased to 3.2 mg/g fresh wt of tissue 5 days after inoculation with *C. fimbriata*, whereas the concentration in untreated and Ethrel-treated carrots increased only to 0.67 and 0.37 mg, respectively. Phenylalanine ammonia-lyase (PAL) activity increased 6-fold in carrots 16 hr after inoculation, whereas the activity in untreated and Ethrel-treated carrots rose 3-fold. The PAL activity decreased in infected tissue after reaching a maximum 16 hr after treatment. Three days after inoculation with *C. fimbriata*, the concentration of MM was twice as high in the phloem as in the xylem of carrot root discs, whereas the chlorogenic acid concentration was equal to or somewhat higher in the xylem than the phloem. Fungitoxic levels of exogenous MM inhibited growth of the fungus on the xylem and phloem.

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Carrot discs inoculated with a spore suspension of the fungus *Ceratocystis fimbriata* Ell. & Halst. rapidly accumulate 6-methoxy mellein (3-methyl-6-methoxy-8-hydroxy-3, 4-dihydroisocoumarin) (MM) (2, 3, 4, 7) and chlorogenic acid (3). The amount of MM that accumulates is fungitoxic to *C. fimbriata* in culture (3, 4). Ethylene also induces accumulation of MM in carrots (1, 2), and the amount accumulated is related to the amount of ethylene produced by the fungus on the carrot disc (2). Sweet potato slices treated with ethylene became resistant to *C. fimbriata*, the incitant of black rot (18). Ethylene causes a rapid accumulation of chlorogenic acid and phenylalanine ammonia-lyase (PAL) in sweet potato tissue (9).

There is a close correlation between the increase in PAL activity and the accumulation of chlorogenic acid in potato tubers (21) and sweet potato roots (9, 11, 12). Zucker (22) demonstrated that PAL activity and chlorogenic acid increased in tubers exposed to light, and protein inhibitors prevented both from increasing. The activity of PAL reached a maximum in 20 to 30 hr after exposure to light, then declined rapidly to one-half the maximum activity. The rapid decline is due to a PAL-inactivating system whose activity is dependent on protein synthesis at the time

of maximum PAL activity. A similar pattern was observed in pea seedling exposed to ethylene; i.e., rapid increase of PAL activity followed by appearance of a PAL-inactivating system (8).

In this study, carrot roots were inoculated with *C. fimbriata* or treated with Ethrel (2-chloroethyl phosphonic acid). Ethrel causes responses in plants characteristic of ethylene gas (5, 6, 10, 16, 19). The experiments were done to determine whether (i) Ethrel induces accumulation of MM and chlorogenic acid; (ii) PAL activity is increased in carrot tissue accumulating large amounts of chlorogenic acid; (iii) there is any difference in accumulation of MM and chlorogenic acid in the xylem and phloem of carrot root; and (iv) metabolic alterations in carrot root leading to the accumulation of MM could be studied in the absence of fungus.

MATERIALS AND METHODS.—*Tissue treatment.*—Carrots (*Daucus carota* L.) purchased locally were used within 2 days of purchase. Carrot roots were washed, surface-sterilized with 75% ethanol for 10 sec, drained, and allowed to dry. They were then cut transversely into discs 1 cm thick under aseptic conditions and randomly distributed into petri dishes. In some experiments, the xylem and phloem were separated before distribution. Discs were sprayed with H_2O (control), a solution of Ethrel (Amchem Products, Inc., Ambler, Pa.), or a spore suspension of *C. fimbriata*, using a sterile chromatogram sprayer, until droplets were apparent on the discs. The suspension of ca. 3×10^6 spores/ml was prepared from a 12-day-old culture. After inoculation, the petri dishes were covered and incubated at 24 C. At the end of each incubation period, the upper millimeter of tissue was removed with a peeler and either extracted or used to prepare acetone powders.

Tissue extraction.—Eight g of tissue were placed in 30 ml of chloroform:methanol (2:1, v/v), homogenized at low speed in a Virtis homogenizer for 3 min, and allowed to stand overnight. The sample was filtered with suction through Whatman No. 5 filter paper, the residue rinsed twice with 10-ml portions of chloroform:methanol, and the combined filtrates were taken to dryness on a rotary evaporator. The residue was dissolved in two 5-ml portions of chloroform:methanol:0.2 N acetic acid (1:2:0.8, v/v) and transferred to a 50-ml test tube, and 2.6-ml portions of chloroform and 0.2 N acetic acid were added. The solution was shaken thoroughly and centrifuged at low speed to separate the two phases. The upper aqueous layer was transferred to a 10-ml volumetric flask and set aside for measurement of chlorogenic acid. The lower chloroform layer was taken to dryness, redissolved in acetone to give a final concentration of 1 g of tissue/ml of solution, and used to measure MM.

MM measurement.—Two hundred μliters of the acetone solution were streaked on a Silica Gel G thin-layer plate and developed in toluene:ethyl formate:formic acid (50:40:10, v/v). MM was detected by its fluorescence under short wavelength ultraviolet radiation, scraped from the plate, and placed in a pasteur pipette plugged with fiberglass. MM was eluted from the silica gel with 95% ethanol into a 5-ml volumetric flask, and the absorbance at 266 nm was measured with a Beckman DB spectrophotometer.

Chlorogenic acid measurement.—The volume of the aqueous extract was adjusted to 10 ml, and the absorbance measured at 325 nm with a Beckman DB spectrophotometer.

PAL measurement.—Acetone powders were prepared by homogenization of tissue in acetone chilled to −20 C with a Virtis homogenizer. The homogenate was filtered through Whatman No. 5 filter paper in a chilled Büchner funnel, and the powder was immediately dried under reduced pressure and stored at −20 C. The PAL extract was prepared by suspending 100 mg of acetone powder in 6 ml of 0.1 M borate buffer, pH 8.8, for 80 min at 2 C. The suspension was centrifuged at 3,000 *g* for 10 min, and the supernatant was used to determine the activity of PAL. Activity was measured by the method of Rahe et al. (14).

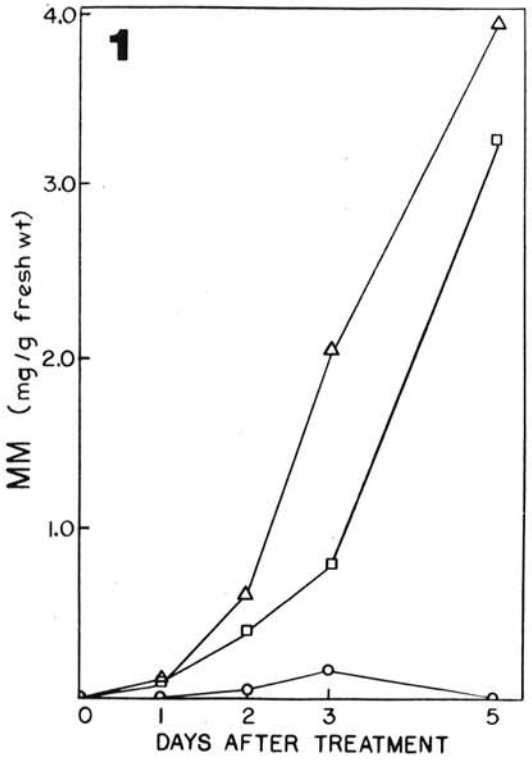
RESULTS.—*MM accumulation.*—The accumulation of MM in the top millimeter of carrot discs treated with a solution containing 9.0 mg Ethrel/ml was equal to or slightly greater than that in discs inoculated with *C. fimbriata* (Fig. 1). The pattern of MM accumulation after treatment with Ethrel or inoculation is very similar. There is a 24- to 48-hr lag period during which MM accumulation is slow, followed by a period of rapid accumulation. MM generally was not detected in fresh carrots and a trace accumulated in aged tissue. The accumulation of the MM increased in carrot discs treated with increasing concentrations of Ethrel up to 9 mg/ml (Fig. 2).

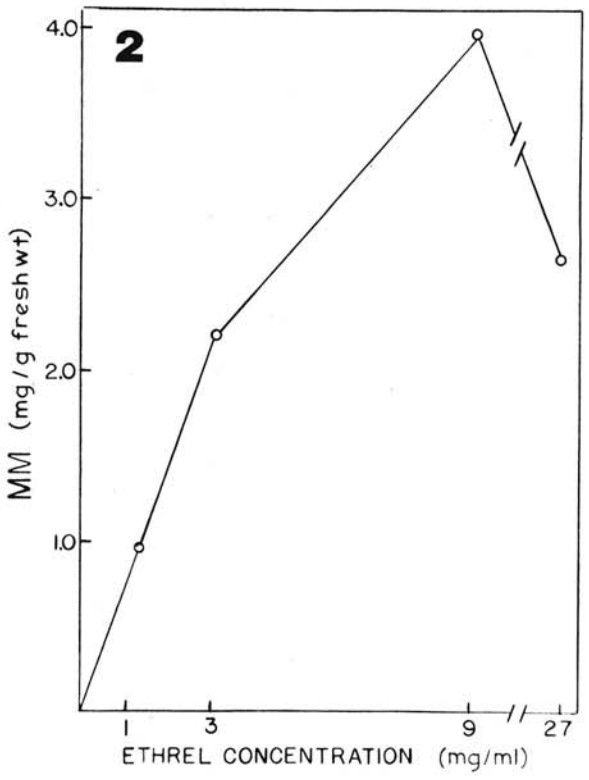
Chlorogenic acid accumulation.—A high level of chlorogenic acid rapidly accumulated in carrot discs inoculated with *C. fimbriata*, whereas less accumulated in water or Ethrel-treated tissue (Fig. 3). The amount of chlorogenic acid increased to 3.2 mg/g fresh wt of tissue 5 days after inoculation with *C. fimbriata*, and the amount in carrots sprayed with water or 9.0 mg/ml Ethrel was 0.67 mg and 0.37 mg, respectively. Chlorogenic acid could not be detected in fresh discs. The initial rate of chlorogenic acid accumulation was approximately the same in the tissue after each of the three treatments. After 24 to 48 hr, there was little or no additional accumulation of chlorogenic acid in carrots treated with water or Ethrel, whereas a rapid rise in accumulation continued in tissue inoculated with *C. fimbriata*. Chlorogenic acid was separated from extracts by thin-layer chromatography on microcrystalline cellulose with 2% aq acetic acid or *n*-butanal:acetic acid:water (4:2:5, v/v). Visual estimation of chlorogenic acid under ultraviolet radiation was consistent with the spectrophotometric determinations.

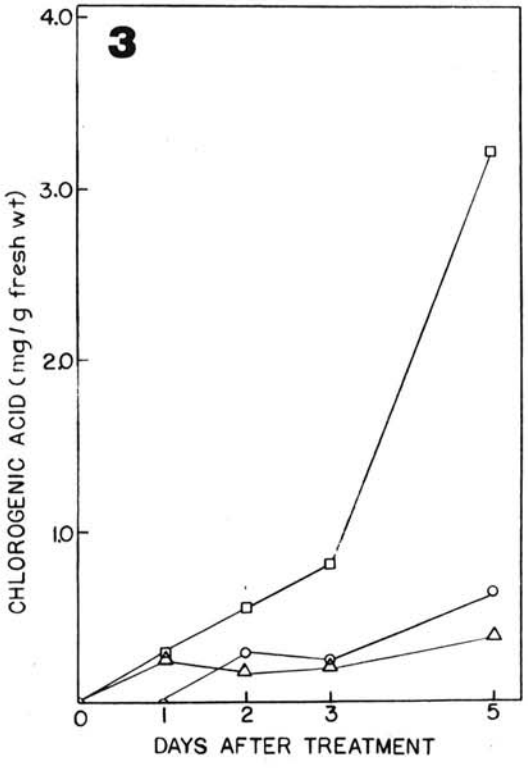
PAL activity.—PAL activity increased to a maximum level approximately 16 hr after the discs were sprayed with water or Ethrel or after inoculation with *C. fimbriata* (Fig. 4). The maximum

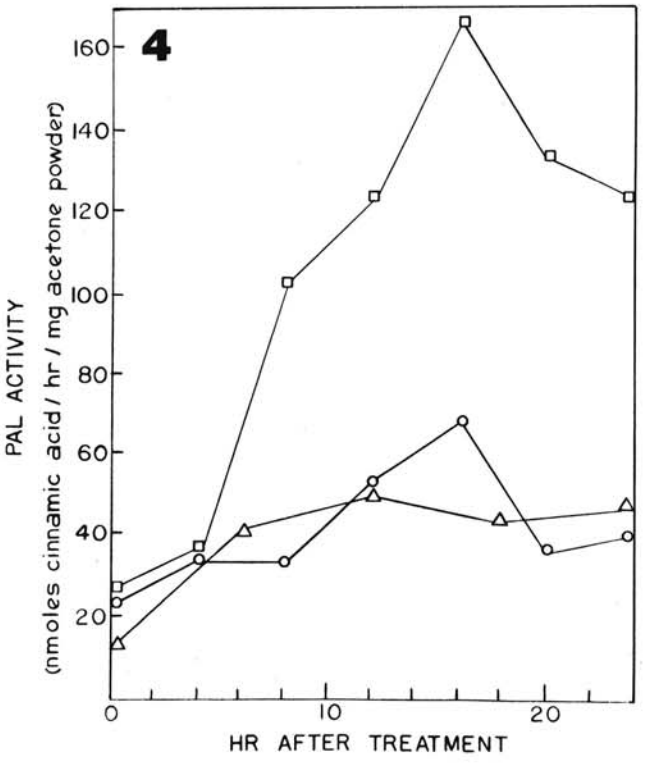
increase in activity in inoculated and noninoculated tissue relative to fresh tissue was 6-fold and 3-fold, respectively. PAL activity decreased after it reached maximum activity, and this decrease was most apparent in the tissue inoculated with the fungus.

Xylem-phloem.—Before inoculating the carrots









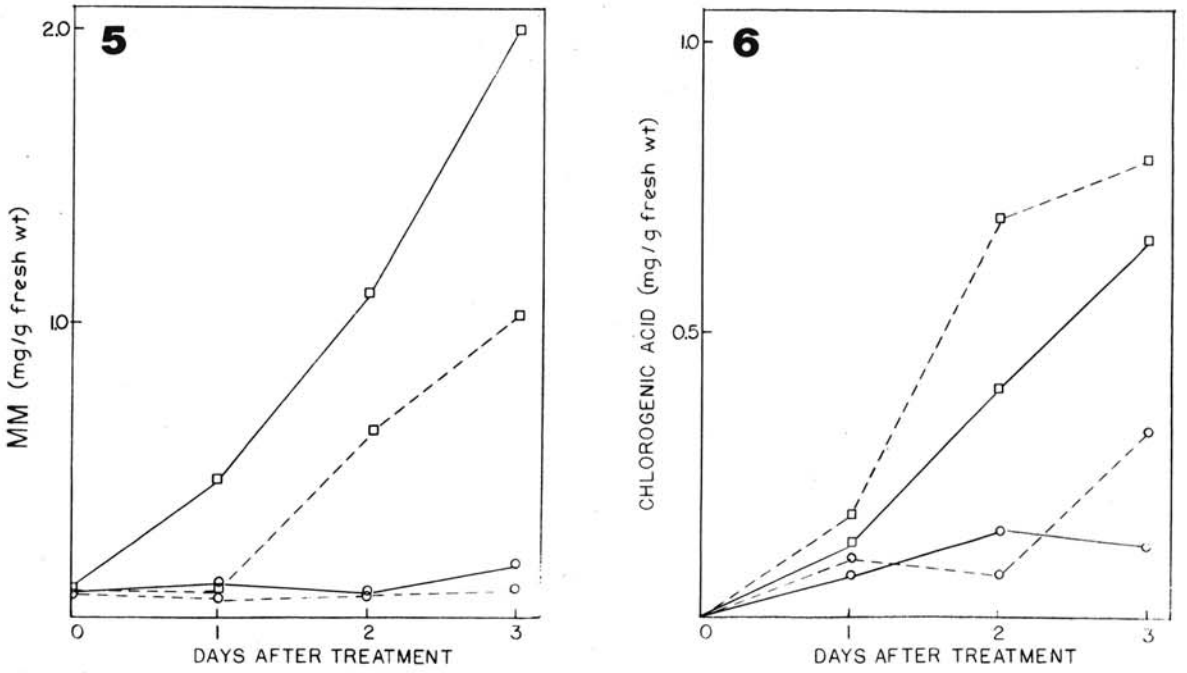


Fig. 5-6. 5) Accumulation of 6-methoxy mellein (MM) in xylem and phloem of carrot root discs; □—□, phloem inoculated with *Ceratocystis fimbriata*; □--□, xylem inoculated with *C. fimbriata*; O—O, phloem treated with H_2O; O--O, xylem treated with H_2O. Data are an average of three experiments. The difference in values from experiments did not exceed 20% of the average. **6)** Accumulation of chlorogenic acid in xylem and phloem of carrot root discs; □—□, phloem inoculated with *C. fimbriata*; □--□, xylem inoculated with *C. fimbriata*; O—O, phloem treated with H_2O; O--O, xylem treated with H_2O. Data are an average of three experiments. The difference in values from experiments did not exceed 30% of the average.

with *C. fimbriata*, we peeled away the phloem from the xylem and each section was treated separately. The tissues were examined for MM and chlorogenic acid accumulation (Fig. 5, 6). The content of MM in the phloem was approximately twice that in the xylem 72 hr after inoculation, whereas the chlorogenic acid was somewhat higher in the xylem. A distinct band of fungus was visible 48 to 72 hr after inoculation on intact disc surfaces in the area of the cambium and secondary xylem cells, and it gradually spread over all the xylem. There was little or no growth of the fungus on the phloem. There was no visible fungus on carrot discs to which 10^{-3} M MM had been applied prior to inoculation, whereas there was no inhibition of growth of the fungus on carrot discs to which 10^{-3} M chlorogenic acid had been applied.

DISCUSSION.—MM accumulates in carrots treated with *C. fimbriata* (3, 4, 7) or ethylene gas (1, 2). The maximum MM accumulating after ethylene treatment is only 25% of that after inoculation with *C. fimbriata* [compare Fig. 1 and Chalutz et al. (2)]. These results have been confirmed in this laboratory (*unpublished data*). Discs treated with 9 mg/ml Ethrel accumulated as much MM as those inoculated with *C. fimbriata* (Fig. 1), and only 1 mg/ml of Ethrel was required to obtain the maximum level of MM accumulating in discs treated with ethylene. Since Ethrel's mode of action appears to be through its decomposition to ethylene gas (20), this increased efficiency may be due to its ability to raise the intracellular level of ethylene by decomposing intracellularly and thus bypassing a rate limiting diffusion of the gas into the cell. If this is so, it indicates that *C. fimbriata* also raises intracellular levels of ethylene or that there are factors in addition to ethylene responsible for induction of MM accumulation by *C. fimbriata*.

Fig. 1-4. 1) Accumulation of 6-methoxy mellein (MM) in carrot root discs treated with: O, H_2O; Δ, 9 mg/ml Ethrel (2-chloroethyl phosphonic acid); □, *Ceratocystis fimbriata*. Data are an average of three experiments. The difference in values from experiments did not exceed 10% of the average. **2)** Accumulation of MM in carrot root discs 5 days after treatment with Ethrel. Data are an average of two experiments. The difference in values from experiments did not exceed 10% of the average. **3)** Accumulation of chlorogenic acid in carrot root discs treated with: O,H_2O; Δ, 9 mg/ml Ethrel; □, *C. fimbriata*. Data are an average of three experiments. The difference in values from experiments did not exceed 20% of the average. **4)** Phenylalanine ammonia-lyase (PAL) activity in carrot root discs treated with: O,H_2O; Δ, 9 mg/ml Ethrel; □, *C. fimbriata*. Data are an average of three experiments. The difference in values from experiments did not exceed 20% of the average.

Ethrel's ability to cause marked accumulation of MM makes it useful for the study of metabolic alterations leading to MM synthesis in carrot root. It permits a study of factors associated with MM metabolism in the absence of both the fungus with its contributing metabolites, and metabolic alterations occuring during the host-parasite interaction that are not directly related to MM synthesis. For example, the accumulation of chlorogenic acid and increase of PAL to the levels found in carrots inoculated with the fungus is not necessary for MM accumulation, since a similar increase is not seen in Ethrel-treated carrots where as much MM accumulated as in the inoculated tissue.

The close relationship of PAL to chlorogenic acid accumulation in potato tuber has been described by Zucker (21). Since chlorogenic acid accumulates in carrot root (3, 17, Fig. 3), the effect of inoculation on PAL activity was also examined. The activity rose rapidly in both control and inoculated tissue (Fig. 4). It was highest in the inoculated tissue that also had the largest accumulation of chlorogenic acid. The PAL activity reached a maximum after 16 hr and then declined. This decline was most evident in the inoculated tissue. The decrease in PAL activity may be due to a lyase-inactivating system that has been demonstrated in both potato tuber and pea seedling (8, 22). In all experiments where PAL and chlorogenic acid were examined, tissue that accumulated more chlorogenic acid also had higher PAL activity. Ethrel had little or not effect on chlorogenic acid accumulation or PAL activity (Fig. 3, 4). Ethylene induces an increase in PAL and chlorogenic acid in sweet potato (9) and an increase in PAL in pea seedling (8) and grapefruit (15). If the MM accumulation in carrots inoculated with *C. fimbriata* is due to the evolution of ethylene, then the carrot must be affected in other ways by the fungus, since Ethrel does not induce the accumulation of chlorogenic acid.

Carrot root can be used as a species-selective medium for isolation of *C. fimbriata* (13). We have observed that the growth of *C. fimbriata* begins in the area of the cambium cells and secondary xylem, then spreads over the remainder of the xylem. There is usually little or no growth of the fungus on the phloem of carrot. The accumulation of MM and chlorogenic acid was examined in separated xylem and phloem of the carrot after inoculation with *C. fimbriata*. MM, which inhibits growth of *C. fimbriata* (2, 3, 4), accumulated in the phloem to levels twice that in the xylem. Chlorogenic acid, which does not inhibit growth of *C. fimbriata* (3), was found in slightly higher concentration in the xylem. Although the MM level is lower in the region that also supports growth of the fungus, the level is sufficient to completely inhibit growth of the fungus in culture (3). The fungus did not grow on a carrot root which had 10^{-3} M MM solution placed on the surface prior to inoculation. Thus, high levels of MM inhibit the fungus in culture or on the carrot. However, if *C. fimbriata* is placed on a carrot with no MM, it will continue to grow even after the MM has accumulated to levels which prevent growth of the fungus in vitro.

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