Increased Disease Resistance and Enzyme Activity Induced by Ethylene and Ethylene Production by Black Rot Infected Sweet Potato Tissue

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Summary. Exposure of root tissue from a susceptible variety of sweet potato to low concentrations of ethylene induced a resistance to infection by Ceratocystis fimбриata and an increase in the activity of peroxidase and polyphenoloxidase in the tissue. Susceptible tissue that was inoculated with a pathogenic strain of C. fimбриata or a nonpathogenic strain that can induce resistance liberated more ethylene into closed chambers than tissue inoculated with strains that did not induce resistance. It is suggested that ethylene may be a stimulus that diffuses from infected areas into adjoining tissue to initiate metabolic changes which may lead to disease resistance. Polyphenol oxidase but not peroxidase activity was increased in slices of potato tubers and parsnip roots treated with ethylene. The activity of these enzymes in root tissue of carrot, radish or turnip was not altered by ethylene treatment.

The disease of sweet potato roots known as black rot is caused by certain isolates of the fungus Ceratocystis fimбриata Ell. and Halst. The surface of root tissue which is normally susceptible to black rot can be made resistant to infection by prior inoculation with nonpathogenic isolates of C. fimбриata (16, 17). Root tissue with this induced resistance has increased peroxidase and polyphenol oxidase activity (16, 17) similar to that found in inoculated naturally resistant tissue. Volatile materials from infected root tissue increased the activity of these enzymes and increased the resistance of uninfected susceptible root tissue (5). Preliminary experiments showed that volatile materials from apples are able to induce similar increases in enzymic activity and resistance in normally susceptible tissue (5). Since apples are known to produce ethylene (2) these results gave support to the view that ethylene may be involved in the changes noted in sweet potato tissue. Ethylene is produced by a variety of injured and infected plant tissues (12) and it was considered that it may be the active volatile material produced by diseased sweet potato tissue.

This paper reports the effect of ethylene on the resistance of sweet potato roots and its effect on the enzymatic activity of roots of sweet potato, parsnip, carrot, turnip, radish and white potato tubers. The production of ethylene by sweet potato root tissue inoculated with pathogenic and nonpathogenic isolates of Ceratocystis is also reported.

Materials and Methods

Preparation of Plant Tissue. Tissues used were white potato tubers (Solanum tuberosum) and roots of sweet potato [Ipomoea batatas (L) Lam.], carrot [Daucus carota (L) var. sativa], parsnip [Pastinaca sativa (L)], turnip [Brassica rapa (L.)] and radish [Raphanus sativus (L)]. The sweet potato varieties were Julian (susceptible to black rot) and Sunny-side (resistant to black rot). Whole roots or tubers were surface sterilized with sodium hypochlorite solution and cut into slices 2 cm thick. These slices were inoculated or treated with ethylene.

Inoculation of Sweet Potato Tissue. Cultures of a pathogenic isolate of C. fimбриata Ell. and Halst. (isolate U-6), another isolate of C. fimбриata (F-38) which is nonpathogenic and induces resistance and a nonpathogen, C. minor (Hedg.) Hunt which does not induce resistance, were grown on potato dextrose agar supplemented with thiamine (14) at 25° for 1 week. Spores from these cultures were suspended in sterile distilled water and used as inoculum. The cut surfaces of sweet potato slices were inoculated with 1 ml of spore suspension. Control slices were treated with sterile distilled water. The slices were placed on stainless steel screens above wet filter paper at the bottom of crystallizing dishes (15 cm × 7.5 cm). Another

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crystallizing dish of the same size was inverted over each dish containing root tissue. The junction between each set of dishes was covered with a strip of aluminum foil and sealed with adhesive tape. Approximately 200 g of tissue was placed in each chamber which had a volume of 2.6 liters. The tissue was incubated at room temperature for up to 6 days.

Gas Analysis. Samples were withdrawn from the chambers described above using a Hamilton gas syringe. Analyses were carried out in an Aerograph 1520 gas chromatography apparatus equipped with a hydrogen flame ionization detector. A stainless steel column (152 cm × 3 mm) packed with 15% diethylene glycol succinate (DEGS) on hexamethyldisilazane (HMDS) treated Chromosorb W (80-100 mesh) was used at 24°. Nitrogen carrier gas was supplied at 15 ml per minute. Hydrogen flow was 32 ml per minute.

Treatment of Tissues with Ethylene. Slices of tissue about 2 cm thick were placed in 20 l containers. Ethylene mixtures of from 8 ppm to 220 ppm were introduced into these chambers using the evacuation method (13). The containers were sealed and incubated at 25° for either 2 or 3 days. Tissues incubated in similar containers in the absence of added ethylene served as controls. The slices were then removed and were either used for the preparation of extracts or were inoculated with spore suspensions of C. fimbriatu and incubated at 25° for 3 weeks. Resistance to infection was assessed after this period.

Preparation of Extracts. Cylinders (18 mm diam) were cut from treated root and tuber slices. Discs 1 mm thick were cut transversely from these cylinders and extracted under nitrogen in an equal volume of 0.1 M Tris-hydrochloride buffer, pH 7.4, containing 0.36 M sucrose (6), and 0.06 M ascorbic acid. Extractions were done at 4° in a modified sodium press. The extracts were centrifuged at 100,000 × g for 15 minutes and the supernatant fractions were collected and stored at −15°.

Gel Electrophoresis. Extracts were subjected to electrophoresis in polyacrylamide gels (10). Gels obtained with extracts of sweet potato were stained to detect amylase, phosphatase, peroxidase and polyphenol oxidase activity. Acid and alkaline phosphatase activity was detected by incubating the gels in filtered solutions of α-naphthyl phosphate (1 mg/ml) diazo blue B (1 mg/ml) and MgCl₂ (1 mg/ml) in either 0.1 M acetic buffer pH 4.6 or 0.1 M Tris HCl buffer pH 9.0 (4). The gels were immersed in the appropriate buffer for 30 minutes before being incubated in the staining solutions. Amylase was detected by incorporating 1% starch in the gels before electrophoresis. After electrophoresis the gels were washed with 0.2 M acetic buffer pH 4.6 for 30 minutes and then incubated in a solution of iodine (0.04 mg/ml), KI (10 mg/ml) and 2 M acetic buffer pH 4.6. Peroxidase was detected using 0.04% solution of 3,3-diaminobenzidine (11). Polyphenol oxidase was detected in gels which had been washed for 30 minutes in 0.1 M phosphate buffer pH 6.5 using a solution of catechol (1 mg/ml) and proline (1 mg/ml) in the same buffer (7).

Enzyme Assays. Peroxidase activity in extracts was measured spectrophotometrically by following the oxidation of pyrogallop at 420 mμ of catechol at 390 mμ (8) at 15 second intervals in the presence of H₂O₂. Polyphenol oxidase was measured spectrophotometrically or manometrically using catechol as substrate (1).

Results

Sweet potato tissue treated with ethylene showed a marked increase in resistance to black rot (fig 1). Extracts of treated tissue showed a marked increase in peroxidase (fig 2) and polyphenol oxidase (fig 3) activity only after electrophoresis in polyacrylamide gels. Polyphenol oxidase activity could not be detected when crude extracts were assayed manometrically or spectrophotometrically. This apparent lack of activity was found to be due to the presence of inhibitory substances in the extracts. The inhibitory substances could be removed by electrophoresis or precipitated from extracts by adjusting them to 1.5 M with ammonium sulfate. Polyphenol oxidase activity was then detected in the fraction which was precipitated with saturated ammonium sulfate. After fractionation with ammonium sulfate, it was found that increases in polyphenol oxidase activity in extracts from ethylene treated tissue could be detected spectrophotometrically (table 1) and manometrically. The results obtained by these 2 methods were similar.

Peroxidase activity in crude extracts and in extracts fractionated with ammonium sulfate were essentially the same when assayed spectrophotometrically. There was an approximate 10-fold increase in peroxidase activity in sweet potato tissues treated with ethylene (table 1).

Extracts from ethylene treated tissue when subjected to gel electrophoresis showed increased amylase activity but no appreciable alteration in acid or alkaline phosphatase activity.

In extracts from white potato treated with ethylene, increased polyphenol oxidase activity was found whereas peroxidase activity decreased (table 1).

Polyphenol oxidase activity was higher in extracts from parsnip roots treated with ethylene than in extracts from untreated roots (fig 3). Polyphenol oxidase activity was detected in extracts only after electrophoresis in polyacrylamide gels. No activity could be detected spectrophotometrically in either crude parsnip extracts or in extracts fractionated with ammonium sulfate. No changes in peroxidase activity of parsnip extracts following ethylene treatment were detected.
FIG. 1. Resistance increased by ethylene treatment. Sweet potato slices were exposed to ethylene-air mixtures for 2 days at the concentrations indicated and then inoculated with *C. fimbriata*. 

Resistance Increased by Ethylene Treatment

Sweet potato slices treated with ethylene for two days and then inoculated with *C. fimbriata*.

Julian

0 ppm 8 ppm 150 ppm
Fig. 2. Peroxidase activity of discs cut 2 mm below the surface of susceptible sweet potato tissue treated with 0 (2), 24 (5), and 220 (8) ppm ethylene. Extracts of discs were subjected to electrophoresis on acrylamide gels and stained for enzyme activity.

Fig. 3. Polyphenol oxidase activity of discs cut 2 mm below the surface of susceptible sweet potato tissue treated with 0 (4), 8 (5) and 150 (6) ppm ethylene. Extracts of discs were subjected to electrophoresis on acrylamide gels and stained for enzyme activity.
Table I. Peroxidase and Polyphenol Oxidase Activity in Extracts of Sweet Potato Root Tissue and White Potato Tuber Tissue Treated with Ethylene

Enzyme activity is expressed as change in optical density at 390 mµ of extract/minute.

<table>
<thead>
<tr>
<th>Ethylene (ppm)</th>
<th>Sweet potato (Julian) Peroxidase</th>
<th>Polyphenol oxidase</th>
<th>White potato Peroxidase</th>
<th>Polyphenol oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.8</td>
<td>0.06</td>
<td>0.20</td>
<td>0.36</td>
</tr>
<tr>
<td>8</td>
<td>20.5</td>
<td>0.13</td>
<td>0.16</td>
<td>1.50</td>
</tr>
<tr>
<td>150</td>
<td>26.8</td>
<td>0.13</td>
<td>0.08</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table II. Ethylene Production by Sweet Potato Tissue Inoculated with Fungal Spores or Left Uninoculated and Incubated for Three Days

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Ethylene produced (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (uninoculated)</td>
<td>1.5</td>
</tr>
<tr>
<td>Ceratocystis minor*</td>
<td>4.0</td>
</tr>
<tr>
<td>C. fimbrina F38***</td>
<td>9.0</td>
</tr>
<tr>
<td>C. fimbrina U6***</td>
<td>15.0</td>
</tr>
</tbody>
</table>

* Nonpathogenic to sweet potato, does not induce resistance to black rot.
** Nonpathogenic to sweet potato, induces resistance to black rot.
*** Produces black rot in susceptible sweet potato.

Ethylene treatment had no apparent effect upon the peroxidase or polyphenol oxidase activity of carrot, radish or turnip root tissue. Very little polyphenol oxidase activity was detected in untreated and ethylene treated radish and turnip extracts.

A volatile compound with the same retention time as ethylene on the gas chromatographic column used in these experiments was demonstrated in the atmosphere surrounding sweet potato tissue incubated in closed chambers after being treated with water or being inoculated with C. fimbrina or C. minor (Table II). The retention time for both ethylene and the material from sweet potato was 20 seconds. In Table II the approximate amounts of this compound produced by the variously treated tissues are expressed as ppm ethylene. The values were obtained by comparing areas under peaks on the chromatograms at a retention time of 20 seconds with areas under peaks produced by known concentrations of ethylene at the same retention time. However, these values and the identification of the compound as ethylene must be regarded as tentative, for only 1 type of column was used. Uninoculated resistant or susceptible tissue produced very little detectable ethylene. Production of ethylene increased following inoculation with all 3 fungi but the increase was larger when the nonpathogenic strain of C. fimbrina (F-38) which induced resistance was used than when the nonpathogenic strain of C. minor which did not induce resistance was used. It was largest when the pathogenic strain was used. Under the experimental conditions used, ethylene production decreased after 4 days incubation. This decrease may have been due to decreased O₂ and increased CO₂ levels within the sealed containers.

Discussion

The results of these experiments indicate that ethylene can increase resistance of sweet potato tissue to infection by C. fimbrina. They also indicate that such induced resistance is associated with an increase in the peroxidase and polyphenol oxidase activity of ethylene treated tissues. Ethylene was not detected above cultures of C. fimbrina grown on potato dextrose agar but was detected above infected sweet potato tissues at concentrations approximating those used in our experimental treatment of uninoculated tissue. It is therefore probable that ethylene is an active principle in volatile materials from infected tissues which cause the increased resistance and peroxidase activity in uninoculated sweet potato tissue (5).

Higher levels of ethylene were observed above sweet potato tissue inoculated with a nonpathogenic strain of C. fimbrina (inducer, F-38) which induces resistance to black rot than above uninoculated tissue or tissue inoculated with nonpathogenic C. minor (noninducer) which does not induce resistance to pathogenic strains of C. fimbrina. This suggests that ethylene may be involved in the induction of resistance to black rot in susceptible tissue inoculated with inducers (16, 17). Peroxidase and polyphenol oxidase activity were also increased in tissue with induced resistance to black rot (16, 17).

It is possible that ethylene is one of the stimuli which move from areas of C. fimbrina infection in sweet potato into adjoining tissue to initiate the metabolic changes which lead to resistance to further penetration by the pathogen. Since ethylene can increase peroxidase and polyphenol oxidase activity in sweet potato tissue it appears that these enzymes may be involved in the resistance mecha-
nism. However, as was previously noted (17), increases in the activity of these enzymes alone cannot be solely responsible for resistance. Other factors are involved and these may include the rate of synthesis or transport of substrates to the areas of increased polyphenol oxidase activity. It is not known whether the inhibitor of polyphenol oxidase found in sweet potato extracts is effective in vivo and hence how much of the increase in activity of this enzyme is effective in the tissue. However, there is a growing body of evidence that peroxidases act in oxidation reactions requiring only catalytic amounts of hydrogen peroxide (18). Increased activity of these 2 enzymes in sweet potato tissue stimulated by ethylene could therefore very likely give rise to abnormal or increased aromatic biosynthesis resulting in the formation of both physical and chemical barriers to infection (9, 15).

Little change in peroxidase or polyphenol oxidase activity was noted in ethylene treated carrot, turnip or radish tissue. Polyphenol oxidase but not peroxidase activity was increased in ethylene treated potato and parsnip tissue. It is possible that the changes noted in ethylene treated sweet potato tissue are peculiar to that species. However, it seems probable that ethylene has a more general role in the defense mechanisms of plants. It may act as a stimulus of localized metabolic changes leading to necrotic and hypersensitive reactions in plants following infection. Since ethylene is capable of producing chlorophyll degradation in fruits (2), it is also possible that ethylene production in infected green plant tissue may cause the chlorosis so commonly observed in such tissues.

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