Induction of Polygalacturonase from *Rhizoctonia solani* by Cotton Seed and Hypocotyl Exudates

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ABSTRACT

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Endopolygalacturonase (EPG) first was detected in 0.5 M NaCl extracts collected from cotton seedling hypocotyls 18 hr after inoculation with *Rhizoctonia solani*. The units of EPG activity in extracts collected 12, 18, 24, 48, and 72 hr after inoculation were 0, 17.5, 36.0, 39.6, and 28.8, respectively. By 18 hr after inoculation, infection cushions had formed, and by 22–24 hr the hypocotyl tissue beneath the cushions was slightly discolored. Neither pectin lyase nor pectate lyase was detected in any of the extracts. The fungus produced extracellular EPG in 0.1% sodium polypectate, in nondialyzed seed exudates, and in dialyzed seed exudates. At 24 hr after seeding with *R. solani*, the units of EPG activity in the above preparations were 28, 4, and 256, respectively. The seed exudates (1 ml/four seeds) were obtained by incubating cotton seeds in water at 22 C for 3 hr. The dialyzed and nondialyzed cotton seed exudates contained about 20 μg of galacturonic acid polymers per milliliter. Solutions obtained by exposing cotton hypocotyls to a commercial pectinase solution for 4 hr or to 0.05 M HCl for 1 hr, induced the fungus to produce 5.6 and 0.4 units, respectively, of EPG activity 24 hr after seeding. This study provides evidence that *R. solani* produces EPG in response to host exudates.

Examination of plant tissues infected by *Rhizoctonia solani* Kühn revealed the presence of pectic enzymes (4,14,17,24). Bateman (7) reported that bean hypocotyls infected with *R. solani* rapidly lost galacturonic acid from their cell walls. Van Etten et al (20) detected enzyme activity in the tissue by 32–36 hr after inoculation. At this time symptoms were visible. Weinhold and Motta (24) observed that cotton seedlings inoculated with *R. solani* lost pectic substances from their cell walls before the pathogen penetrated the cuticle. Thus pectolytic enzymes produced by the fungus before penetration may aid the pathogen in penetrating the host plant. It therefore seems important to determine the relationship of the production of pectolytic enzymes by *R. solani* to symptom development.

Considerable evidence exists that *R. solani* produces polygalacturonase inductively (2,5,9). This fact plus the observation that cell wall degradation occurs while the pathogen is on the surface of the host (24) suggest that *R. solani* produces polygalacturonase in response to substances available to the pathogen before penetration. Host exudates are a possible source of such substances. Cotton seed exudates have been implicated as a source of nutrients for the growth of *R. solani* to the host and for the formation of infection structures (12,23). The role of exudates in inducing cell wall-degrading enzymes has not been explored. Because *R. solani* will attack seedlings in the absence of seed exudates (23), polygalacturonase-inducing substances may be available to the pathogen from the surface of the hypocotyl. This study investigated the production of polygalacturonase from *R. solani* during the initial stages of host-pathogen interaction, with emphasis on the role of host exudates as a source of enzyme-inducing substances.

MATERIALS AND METHODS

The isolates of *R. solani* used in this study were from cotton in the San Joaquin Valley, CA, and belong to anastomosis group 4. Procedures for growing the fungus in culture to produce inoculum and for inoculating cotton seedlings (*Gossypium hirsutum* L.) in the laboratory have been described (22).

Extraction of pectolytic enzymes from inoculated seedlings. Attempts were made to extract pectic enzymes from cotton seedlings at 12, 18, 24, 48, and 72 hr after inoculation. Segments 1.8 cm long were cut from the inoculation site of seedlings incubated in three replicate plates. Extracts were prepared by grinding the 15 segments from each plate for 1 min at 4 C in 5 ml of 0.5 M NaCl with a VirTis homogenizer. Each of the three extracts was strained through three layers of cheesecloth and centrifuged at 6,000 *g* for 20 min at 4 C. The supernatant fluids were dialyzed overnight at 4 C and stored at −18 C.

Collection of cotton seed and hypocotyl exudates. Exudates were obtained by placing 12.6 g of cotton seeds (100 ± 3 seeds) in each of 50 petri dishes containing 15 ml of distilled water. After 3 hr at 22 C, the liquid in the dishes was filtered through a 0.22 μm millipore filter and the volume was adjusted to 25 ml per 100 seeds. This solution represents the nonfractionated seed exudates. One hundred milliliters of this exudate solution was dialyzed overnight against 4 L of distilled water at 4 C and labeled the dialyzed seed exudate. Sixty milliliters of the nonfractionated exudate solution was added to 240 ml of absolute ethanol. The resulting precipitate was collected by centrifugation at 15,000 *g* for 15 min at 4 C. The supernatant solution was decanted, taken to dryness under reduced pressure at 45 C, resuspended in 50 ml of distilled water, and labeled the ethanol soluble fraction. The precipitate was dissolved in 25 ml of distilled water, heated at 45 C under reduced pressure until dry, resuspended in 50 ml of distilled water, and labeled the ethanol insoluble fraction. The exudate solutions were filtered through 0.22 μm Millipore filters.

Exudates also were collected from the hypocotyls of 6-day-old cotton seedlings to determine if they could induce *R. solani* to produce pectolytic enzymes. Twenty cotton seedlings, grown in the greenhouse, were removed from the planting mix and held in a "U" shape around the outside of a test tube. The seedlings were positioned so that the hypocotyls were at the bottom of the tube with the leaves and roots along the sides. A rubber band was stretched around the roots and stems to hold the seedlings in place. Three exudate solutions were obtained by placing the hypocotyl portion of the seedlings in 30 ml of water, 0.05 M HCl, or 0.05 mg/ml pectinase (Sigma Chemical Co., St. Louis, MO 93178). After 1 hr the hypocotyls in the 0.05 M HCl solution were removed, and after 4 hr the hypocotyls in the pectinase solution and water were removed. The 0.05 M HCl solution was adjusted to pH 7.0 with NaOH and the three exudate solutions were filtered through 0.22 μm Millipore filters.

Production of pectolytic enzymes by *R. solani* in culture. Seed exudates, hypocotyl exudates, and sodium polypectate (1 mg/ml)

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were used to induce *R. solani* to produce pectolytic enzymes in culture. Mycelial discs, 2 mm in diameter, were cut from 3-day-old cultures of *R. solani* grown on a liquid medium (22). Twenty discs were placed in 25-ml Erlenmeyer flasks, each containing 10 ml of sterile distilled water. The flasks were placed on a water bath shaker at 28 C and 40 oscillations per minute. After 18 hr, the water was decanted, 10 ml of the inducer solution to be tested was added to each flask, and the flasks were returned to the shaker. One-milliliter samples of the liquid in each flask were removed at various intervals and were assayed for pectolytic enzyme activity.

Pectolytic enzyme assay. To detect hydrolytic enzymes, 5 ml of 1% sodium polypectate or pectin NF in 0.1 M sodium acetate buffer (pH 5.2) was added to a size 300 Ostwald-Fenske viscometer in a water bath at 28 C. Pectate lyase and pectin lyase activities were measured similarly, except that 0.1 M tris-HCl buffer (pH 8.4) containing 0.12 mM $CaCl_2$ was substituted for the acetate buffer. For the viscometric assays, units of enzyme activity were determined by dividing 1,000 by the minutes required for 1 ml of enzyme preparation to reduce the viscosity by 50%. Pectase lyase and pectin lyase activities also were measured by direct spectrophotometric analysis of reaction mixtures at 232 nm (1), and by the periodate-thiobarbituric acid procedure (18).

The number of reducing groups liberated by the action of polygalacturonase was determined by the dinitrosalicylic acid procedure (15). Four milliliters of 0.08% polygalacturonic acid in 0.02 M sodium acetate buffer (pH 5.2) was incubated with 1 ml of enzyme preparation at 28 C. The enzyme activity was expressed in units defined as μmoles of galacturonic acid released per minute per milliliter of enzyme preparation.

Assay for galacturonic acid in seed exudates. The uronate dehydrogenase procedure was used to detect galacturonic acid in untreated exudates and in exudates treated with pectinase (8). Monomers, but not polymers, of galacturonic acid can be detected by this assay procedure. Commercial pectinase (Sigma Chemical Co.) was added to convert the polymers of galacturonic acid to monomers. To estimate the polygalacturonic acid content in the exudates, the difference in galacturonic acid content of pectinase-treated exudate samples and untreated samples was determined. Thirty-five milliliters of exudate solution was placed in a boiling water bath for 10 min. After cooling, 3.5 ml of 1 M sodium acetate buffer (pH 4.0) was added. Five milliliters of buffered exudate and one drop of toluene were placed in each of six flasks. To three of the flasks, 0.8 ml of dialyzed pectinase (1 mg/ml) was added. The same amount of pectinase, which had been inactivated by heating at 121 C for 15 min, was added to the other three flasks. The buffer, exudate, and inactivated pectinase in these three flasks represented the untreated exudate reaction mixture. After 24 hr at 25 C, 2-ml samples were removed from each flask, adjusted to pH 8.0 with NaOH, and then divided equally between two test tubes, each containing 100 μl of 1 M tris-HCl buffer (pH 8.0). To one of these tubes, 10 μl of 0.014 M nicotinamide adenine dinucleotide (NAD), 50 μl of water containing 0.03 units of uronate dehydrogenase (UAD), and enough distilled water to bring the volume to 2 ml were added. The second tube was treated similarly, except that the UAD was omitted. After 45 min at 23 C, the absorbance was measured at 340 nm; the tube without UAD was used as the blank. Solutions containing from 10 to 100 μg of galacturonic acid per milliliter were used as standards. The UAD was obtained from T. Kosuge (University of California, Davis). One unit of this enzyme was defined as the amount required to catalyze the reduction of 1 μmole of NAD per minute, when 100 μg/ml of galacturonic acid was substituted for the exudate samples. The results are expressed as micrograms of galacturonic acid per milliliter of exudate.

Paper chromatography was used to confirm the presence of galacturonic acid polymers in cotton seed exudates. After the pectinase treatment, the exudate samples were applied to columns (1×10.5 cm) of Bio-Rad Ag 1 anion exchange resin (formate form). After being washed with 25 ml of distilled water, the galacturonic acid was eluted from the columns with 1 N formic acid. The first 20 ml of formic acid eluate was reduced to 2 ml under reduced pressure at 40 C. Fifty microliters of this concentrated solution was applied to Whatman No. 1 paper. One-dimensional paper chromatography was used and the solvent system consisted of 85 ml of 77% ethanol and 15 ml of 88% formic acid (16). A bromophenol indicator solution was used as a spray (16).

RESULTS

Pectolytic enzyme activity in cotton seedlings in relation to infection by *R. solani*. The pectolytic enzyme assays were performed on dialyzed extracts obtained from cotton hypocotyls at 12, 18, 24, 48, and 72 hr after inoculation. Extracts from noninoculated cotton seedlings also were assayed for pectolytic enzyme activity. Each experiment was repeated four times with three replications. For each replication, the enzyme assays were performed on dialyzed extracts from 15 cotton seedlings.

Extracts prepared 12, 18, 24, 48, and 72 hr after inoculation contained 0, 17.5, 36.0, 39.6, and 28.8 units of pectolytic activity, respectively (Fig. 1). No pectolytic activity was detected when viscometry was performed on extracts from noninoculated seedlings. Pectolytic activity was about four times greater with sodium polypectate than with pectin N.F. solutions (Fig. 1), suggesting that polygalacturonase was present in extracts prepared from cotton seedlings at 18, 24, 48, and 72 hr after inoculation. There was 0.5% hydrolysis of substrate at 50% reduction in viscosity, which indicated that the polygalacturonase attacked internal linkages (Fig. 1). Paper chromatography of reaction mixtures containing polygalacturonic acid and an extract prepared 18 hr after inoculation confirmed that the enzyme released polymers rather than monomers of galacturonic acid as the initial reaction products. Viscometric, direct spectrophotometric, and periodate-thiobarbituric acid assay procedures showed no lyases in extracts prepared from cotton seedlings at 12, 18, 24, 48, and 72 hr after inoculation. These results demonstrated that endopolygalacturonase (EPG) was present in cotton hypocotyls within 18 hr after inoculation with *R. solani*.

Cotton seedlings were examined at various intervals after inoculation to determine whether the EPG was produced in time to cause the symptoms observed. At 18 hr after inoculation, infection cushions were present on cotton hypocotyls. Initial symptoms, consisting of tissue discoloration beneath infection cushions, appeared at 22–24 hr after inoculation. By 26–28 hr, lesions with macerated tissue were present.

Induction of polygalacturonase from *R. solani* by seed and hypocotyl exudates. Samples collected 12, 24, 48, and 96 hr after

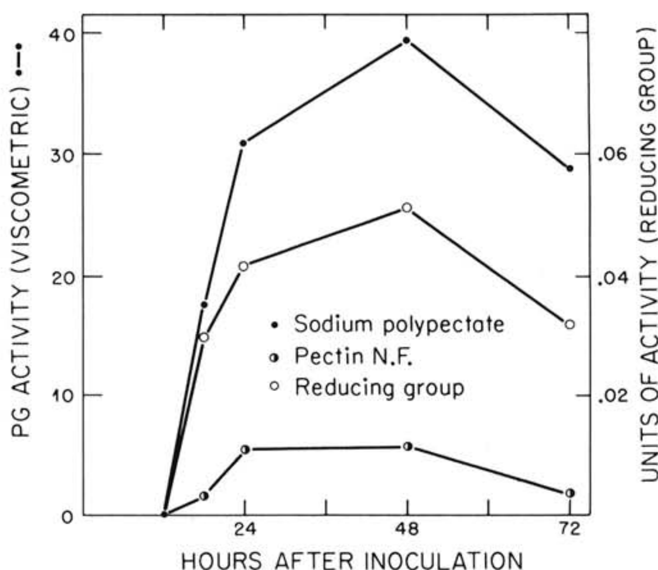

Fig. 1. Polygalacturonase (PG) activity in 0.5 M NaCl extracts from cotton hypocotyls prepared at various times after inoculation with *R. solani*. Activity was determined by the viscometric method using sodium polypectate and pectin N.F. as substrates and by the dinitrosalicylic acid-reducing group method with sodium polypectate as the substrate.

the fungus was exposed to the nonfractionated seed exudates contained 0, 4, 239, and 471 units of EPG activity, respectively (Table 1). When exudates were dialyzed before they were added to the cultures, samples collected at these intervals contained 248, 256, 360, and 276 units of EPG activity, respectively. No trans-eliminases were detected.

The fungus produced EPG when exposed to the 80% ethanol insoluble fraction of seed exudates (Table 1). Fifteen units of polygalacturonase activity were detected in samples collected 12 hr after the fungus was exposed. The polygalacturonase activity in samples from flasks containing ethanol insoluble exudates remained at about 20 units after 24, 48, and 96 hr exposure and did not reach the high levels detected when nonfractionated or dialyzed exudates were used. The fungus also produced EPG in response to the 80% ethanol soluble fraction, but no activity was detected until the fungus was exposed to this material for 48 hr (Table 1). Even after 96 hr, the EPG activity was only 17 units. The fungus also produced EPG in response to 0.1% sodium polypectate (Table 1). Within 12 hr after this material was added to the fungus, 37 units of EPG were detected. The EPG activity in samples from flasks containing sodium polypectate decreased after 12 hr, and by 96 hr no EPG activity could be detected.

The fungus produced EPG in respone to exudates collected when cotton hypocotyls were placed in a solution of 0.05 mg/ml of commercial pectinase for 4 hr. The liquid collected from flasks 24 and 48 hr after the fungus was exposed to this exudate solution contained 5.6 and 6.4 units of EPG activity, respectively. The fungus produced small amounts of EPG in response to the exudate solution collected by placing hypocotyls in 0.05 M HCl for 1 hr. After the fungus was exposed to this exudate solution for 24 and 48 hr, the samples from flasks contained 0.4 and 1.1 units of EPG activity, respectively. The fungus did not produce detectable amounts of EPG when exposed to the exudate solution obtained by placing cotton hypocotyls in water for 4 hr.

Galacturonic acid and soluble carbohydrate content of cotton seed exudate. The nonfractionated, dialyzed, and ethanol insoluble fraction of seed exudates that had been exposed to pectinase contained 24.2, 21.9, and 20.6 μg of galacturonic acid per milliliter, respectively (Table 2). The pectinase-treated, ethanol-soluble fraction contained 6.1 μg/ml of galacturonic acid.

Paper chromatography of the dialyzed exudate solutions confirmed the presence of galacturonic acid polymers. Galacturonic acid could not be detected in the dialyzed exudate solution when the pectinase treatment was omitted.

TABLE 1. Polygalacturonase production by *Rhizoctonia solani* in response to cotton seed exudates

	Polygalacturonase activity units[a] after exposure of the fungus to inducer for (hr):			
Fraction tested	12	24	48	96
Nonfractionated exudates	0	4	239	471
Dialyzed exudates	248	256	360	276
Ethanol-insoluble fraction	15	18	22	20
Ethanol-soluble fraction	0	0	4	17
Sodium polypectate (0.1%)	37	28	8	0

[a] Polygalacturonase activity per milliliter of filtrate was determined by the viscometric method (pH 5.2). Unit = 1000/t; t = time in min for 1 ml of sample to reduce the viscosity of 5 ml of 0.96% sodium polypectate by 50%. The viscometric method is nonspecific and thus results cannot be interpreted in a quantitative manner. Each value represents the mean of three experiments with three replications per experiment.

TABLE 2. Galacturonic acid content of cotton seed exudates

	Galacturonic acid (μg/ml)	
Exudate fraction	Polymers and monomers	Monomers only
Nonfractionated	24.2[a]	4.1
Dialyzed	21.9	0.9
Ethanol insoluble	20.6	1.1
Ethanol soluble	6.1	3.0

[a] Each value represents the mean of three experiments with two determinations on each exudate sample per experiment.

DISCUSSION

EPG consistently was detected in extracts obtained from cotton hypocotyls 18 hr after inoculation with *R. solani*. At this stage of the infection process, infection cushions were formed, but macroscopic symptoms did not appear until 4–6 hr later. This early production of EPG coincides closely with Weinhold and Motta's observation that pectic substances were removed from cotton hypocotyl cell walls about 12 hr before the pathogen penetrated the cuticle (24). Other plant pathogens also produce EPG as the first of a series of cell wall-degrading enzymes (10,13).

Our results provide evidence that EPG is responsible for the tissue maceration associated with infection of cotton seedlings by *R. solani*. Neither pectin lyase nor pectate lyase was detected in any of the extracts obtained from cotton seedlings at various times after inoculation. Neither of these trans-eliminases was produced when the fungus was exposed to cotton seed exudates or sodium polypectate in culture. These results contrast those that indicate production of a pectolytic trans-eliminase in hosts other than cotton after *R. solani* infection (2,6,19) and in culture. Our study shows that EPG is produced in time to cause the tissue maceration associated with infection of cotton seedlings by *R. solani*. If pectin lyase or pectate lyase are involved in the infection of cotton seedlings, they probably are involved in the later stages of pathogenesis.

Several reports indicate that *R. solani* produces EPG inductively (2,5,9). Gupta (11), however, reported that an *R. solani* isolate produced EPG in the absence of pectic substances. The *R. solani* isolate used in the present study produced EPG in culture only when incubated with pectic substances. EPG production by this *R. solani* isolate is repressed by glucose and other readily usable substrates (21).

Because EPG was extracted from cotton hypocotyls inoculated with *R. solani* before the pathogen penetrated the cuticle, the enzyme must be produced in response to substances available to the pathogen prior to penetration. A similar situation was reported by Barash et al (3). They demonstrated that safflower blossom exudates contained pectic substances and induced *Botrytis cinerea* to produce polygalacturonase in culture. Our results show that cotton seed exudates contain polygalacturonic acid and that *R. solani* can produce EPG in response to these exudates. Exudates released from cotton hypocotyls also are a possible source of pectic substances. Because *R. solani* can produce EPG in response to exudates obtained by treating cotton hypocotyls with dilute acid or pectinase solutions, it is likely that the pathogen is capable of obtaining inducing substances from cotton hypocotyls.

Our results suggest that host exudates, in addition to providing nutrients for growth and the formation of infection structures, also play an important role in the infection process by inducing *R. solani* to produce EPG.

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