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Purification and Characterization of Extracellular Pectinesterases from *Phytophthora infestans*

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ABSTRACT

Constitutively produced extracellular pectinesterases from culture filtrates of the potato late blight fungus *Phytophthora infestans* were purified and characterized. One enzyme (PE II) was purified to homogeneity. Sodium dodecyl sulfate electrophoresis of the second enzyme (PE I) revealed two protein bands; there are indications that both proteins are pectinesterases, which were not separable by a number of different techniques. Thus, *P. infestans* might produce three pectinesterases *in vitro*. Enzyme activities were optimal in the neutral pH range and were largely dependent on the presence of NaCl or $CaCl_2$ in the reaction medium. The molecular weight of the PE I-complex was between 45 and 48 kilodaltons, and the one of PE II was between 35 and 40 kilodaltons. Further investigations will help us to clarify the role of these enzymes during pathogenesis.

The following pectolytic enzyme activities have been found in culture filtrates of *Phytophthora infestans*: pectinesterase (EC 3.1.1.11 [2, 6, 10]), endo-polygalacturonase (EC 3.2.1.15 [3, 10]), and galactanase (EC 3.2.1.89 [3, 10–12]). Only one galactanase and an endo-polygalacturonase have been partially purified (3, 10).

The significance of pectinesterases in pathogenesis is unclear and speculative. Demethylation of pectin may be a prerequisite for the action of chain splitting enzymes like polygalacturonases and pectate lyases. On the other hand, free carboxyl groups formed by the action of PE[1] might react with Ca or other multivalent cations to form rigid cross connections between pectin chains. Incorporation of Ca into the pectic fraction appears to make the cell walls increasingly resistant to hydrolysis by cell wall degrading enzymes and to physicochemical changes (17). Also, pectic enzymes might induce host reactions by releasing elicitor-active carbohydrates from plant cell walls. Thus, they may be responsible for phytoalexin production and play a role in plant disease resistance to microorganisms (4, 14).

As a prerequisite to study the significance of pectinesterases during pathogenesis at the host-parasite interface *in vivo*, we have been purifying these enzymes from culture filtrates of *P. infestans*.

MATERIALS AND METHODS

Organisms and Production of Crude Enzyme Extract. Stock cultures of *P. infestans* (race 4) were grown on rye agar at 16°C. To maintain its pathogenicity, the fungus was reisolated from potato tubers (*Solanum tuberosum*, variety 'Bintje') every 3 to 4 months. For enzyme production, *P. infestans* was grown in a nutrient broth (8) in Roux bottles at 20°C for 2 weeks. The culture filtrate served as source for enzyme extraction.

To test a stimulation of PE production by pectin, 1% or 0.1% (w/v) pectin was added to the culture medium. Since pectin solutions are degraded by autoclaving, pectin was sterilized separately in Erlenmeyer flasks by heating at 120°C for 6 h. It was then dissolved in sterile distilled H_2O, neutralized by adding sterile NaOH, and added to the nutrient broth.

Enzyme Assays. The reaction mixture for the pectin esterase assay consisted of 1.5 ml 2% (w/v) pectin (pectin from citrus; Serva) in 0.25 M NaCl (pH 7), 1.5 ml 10 mM Na-phosphate buffer (pH 6.5) and enzyme extract; the total volume was 3.5 ml. The enzyme activity was assayed by measuring the pH decrease within 30 min at 22°C. With the enzyme quantities tested, the pH decrease was linear within that time. One unit of enzyme activity was defined as the amount of enzyme which caused a decrease in pH of the reaction mixture of 0.1 in 30 min.

The effect of salts on PE activity was determined by adding concentrated solutions of NaCl or $CaCl_2$ to the reaction medium giving final concentrations of 5×10^{-1}, 10^{-1}, and 10^{-2} M NaCl and 10^{-1}, 10^{-2}, and 10^{-3} M $CaCl_2$. The pectin solution was prepared without adding NaCl in this case.

For determination of the pH optimum of PE activity, solutions of 2% (w/v) pectin in 0.25 M NaCl were adjusted with NaOH to pH 5, 6, 7, 8, or 9. One hundred µl purified enzyme extract was added to 9 ml pectin solution. The reaction mixture was stirred continuously. Enzyme activity was determined by titration with 0.015 N NaOH of the carboxyl groups released during the enzyme reaction. Activity was expressed by the amount of NaOH consumed within 30 min at 22°C.

Pectate lyase and pectin lyase activities were determined photometrically by measuring the increase of adsorbance at 235 nm (1, 15) extending the incubation time to 1 h. Enzyme extracts equivalent to 40 units PE I or PE II (pectinesterases I and II) were tested.

Polygalacturonase activity was determined by measuring the increase of reducing groups in the reaction mixture according to Nelson (19) after 1 h at 25°C. The reaction mixture contained 2.5 ml 0.5% pectic acid (pH 5), 2.5 ml 0.1 M citrate-phosphate buffer (pH 5), and 0.5 ml enzyme extract equivalent to 50 units PE I or PE II.

Enzyme Purification. All procedures were carried out at 4°C. Culture filtrate (4 L portions) was lyophilized and dissolved in 200 ml distilled H_2O. After dialysis against 10 mM Na-phosphate buffer (pH 6.5) for 24 h, EDTA was added to give a final concentration of 10^{-4} M in the crude extract. The extract was brought to 50% saturation with $(NH_4)_2SO_4$, and after 1 h of stirring it was centrifuged at 48,000*g* for 45 min. The precipitate

[1] Abbreviation: PE, pectinesterase.

containing no pectinesterase activity was discarded. The supernatant was brought to 90% saturation with $(NH_4)_2SO_4$, and after 1 h of stirring was centrifuged again for 1 h at 48,000*g*. The precipitate was collected, redissolved in distilled H_2O, and dialyzed against 10 mM Na-phosphate buffer (pH 6.5) for 24 h. The dialyzed solution was passed through a 5 × 12 cm column of DEAE-cellulose (Whatman DE 52) equilibrated and eluted with the above buffer. Enzyme activity was eluted, whereas some other protein and pigmented material was adsorbed to the packing. The enzyme extract from the DEAE-column was lyophilized to dryness, redissolved in distilled H_2O, and dialyzed against 10 mM Na-phosphate buffer (pH 6.5) for 24 h. It was then applied to a CM-cellulose (Whatman CM 52) column (3.5 × 10 cm), equilibrated with the same buffer. The column was washed with 70 ml equilibration buffer and was then eluted with a linear gradient of 0 to 0.2 M NaCl in 10 mM Na-phosphate buffer (pH 6.5) with a flow rate of 70 ml/h. Fractions containing PE I were combined and processed for chromatofocusing: the extract was concentrated on a PM 10 membrane (Amicon) and by collodion bags (Sartorius), and dialyzed against 25 mM ethanolamine-CH_3COOH buffer (pH 9.5) for 36 h. The extract was applied to a 1 × 30 cm column of polybuffer exchanger PBE 94 (Pharmacia) equilibrated with the above buffer. The column was eluted with 125 ml polybuffer 96 (Pharmacia) diluted 1:10 (pH 7.5). The flow rate was 10 ml/h; 5-ml fractions were collected. Active fractions were combined, concentrated in a dialysis bag using PEG 20,000 (Serva) and afterwards in a collodion bag (Sartorius). It was then applied to a 1 × 100 cm column of Sephacryl S-200 Superfine (Pharmacia) equilibrated and eluted with 50 mM Na-phosphate buffer (pH 6.5). The flow rate was 2.5 ml/h.

Active fractions from CM-cellulose chromatography containing PE II were combined, lyophilized, redissolved in distilled H_2O, and run on Sephacryl S-200 under the same conditions as with PE I.

SDS-PAGE. Slab gel electrophoresis was carried out according to Laemmli (13) and stained with either Coomassie blue R 250 (21) or silver nitrate (18).

Mol Wt Determination. Mol wt was determined either by gel chromatography with Sephacryl S-200 Superfine using lactate dehydrogenase, BSA, ovalbumin, chymotrypsinogen, lysozyme, and Cyt *c* as protein standards, and dextran blue as void volume marker or by SDS-PAGE with the following protein standards: phosphorylase B, BSA, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

Assay for Glycoproteins. Glycoproteins were determined on SDS-PAGE gels with a modified silver staining procedure according to Dubray and Bezard (5) except that the incubation time in 0.2% periodic acid was only 30 min. Additionally, the adsorption of PE I to Con A-Sepharose (Sigma) was tested. The starting conditions were: 10 mM Na-phosphate buffer, 0.5 M NaCl, (pH 6.5). The elution was carried out by a gradient of 0 to 1 M α-methyl mannoside dissolved in starting buffer.

Table I. *Purification of Extracellular Pectinesterases of P. infestans*

	Specific Activity		Recovery	
	units/mg protein		%	
Culture broth	13		100	
Ammonium sulfate precipitate	32		92	
DEAE-cellulose	525		86	
	PE I	PE II	PE I	PE II
CM-cellulose	1,585	1,745	61 = 40 + 21	
Chromatofocusing	—[a]		28	
Sephacryl S-200: total activity peak			30 = 24 + 6	
Sephacryl S-200: pure fractions	12,100	15,350	12 = 11 + 1	

[a] Not determined since polybuffer interferes with the Lowry protein method.

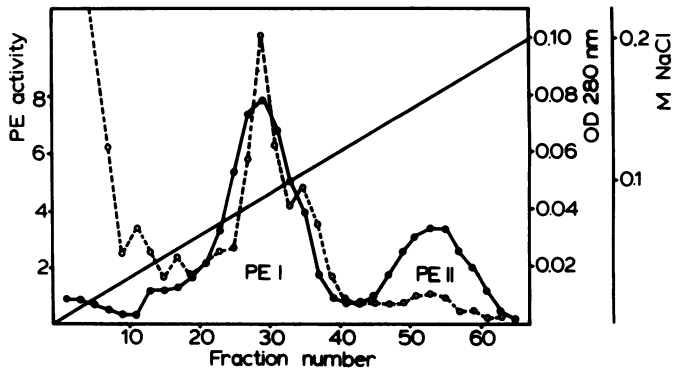

FIG. 1. CM-cellulose chromatography. The column (3.5 × 10 cm) was equilibrated with 10 mM Na-phosphate buffer (pH 6.5). The lyophilized and dialyzed enzyme extract from DEAE-cellulose chromatography was applied to the column, the column was washed with 70 ml of the equilibration buffer and was then eluted with a linear gradient of 0 to 0.2 M NaCl in 10 mM Na-phosphate buffer (pH 6.5) at a flow rate of 70 ml/h. The total volume of the gradient was 500 ml. Fractions of 8 ml were collected and assayed for PE activity and absorbance at 280 nm. (●——●), PE activity (units/50 μl); (O- - -O), A_{280nm}.

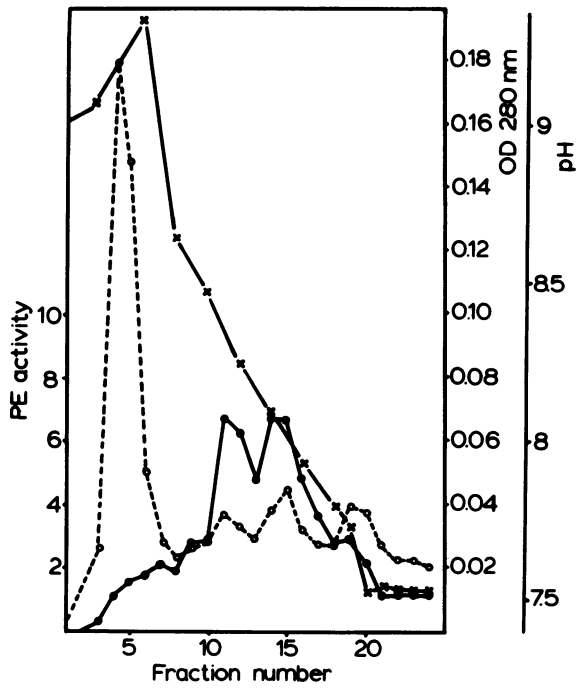

FIG. 2. Chromatofocusing on polybuffer exchanger PBE 94 of CM-cellulose-purified PE I extract. The column (1 × 30 cm) was equilibrated with 25 mM ethanolamine-CH_3COOH buffer (pH 9.5) and eluted with 125 ml polybuffer (pH 7.5). Five-ml fractions were collected at a flow rate of 10 ml/h. (●——●), PE activity (units/50 μl); (O- - -O), $A_{280\ nm}$; (×——×), pH.

RESULTS

Fungus Culture and Enzyme Production. After 2 weeks growth on Hohl's nutrient broth, the culture medium was covered with

a thick mycelial mat of *P. infestans*. The culture filtrate contained approximately 2 units PE activity/ml. Enzymes were produced constitutively. Adding 1% or 0.1% pectin to the nutrient medium did not stimulate enzyme production.

Enzyme Purification. Results of the enzyme purification are summarized in Table I. The enzymes were purified about 1000-fold; the recovery of total enzyme activity was about 12%.

PE did not adsorb to DEAE-cellulose and was eluted with the starting buffer. Chromatography on CM-cellulose and elution with a NaCl-gradient revealed that total PE activity was based on two enzymes, further referred to PE I and PE II (Fig. 1). PE I was eluted first and occurred in larger amounts than PE II. Protein peaks coincided with PE activity peaks, but SDS electrophoresis showed that both enzyme preparations were impure.

Chromatofocusing of the PE I extract removed further impurities (Fig. 2). At a starting pH of 9.5, PE I was adsorbed to the polybuffer exchanger and was desorbed at the pH range between 8.6 and 7.5, showing that the isoelectric point of PE I is within this range. The activity peak always consisted of two not completely separable peaks, supposing that PE I consists of two different proteins.

Chromatofocusing of PE II, which is not shown here, under the same conditions, showed that the isoelectric point of this protein is above the one of PE I: PE II was not adsorbed to the exchanger at pH 9.5.

Gel filtration of the PE I extract from chromatofocusing had an elution profile as shown in Figure 3. SDS-PAGE of the left part of the peak showed only two protein bands (Fig. 5a, lane 1). Confidently, the lower protein band represents a PE. We assume that the upper band is another PE. This is substantiated by the facts that (a) we got a double activity peak in chromatofocusing and (b) it was impossible to separate the double peak by gel filtration; but SDS-electrophoresis of the single Sephacryl fractions when equal PE activities were applied to the gel revealed that, in the first peak fractions, the upper protein band dominated, whereas in the last fractions there was only the lower band present. Repeating the whole purification procedure several times, in some cases, there was a faint impurity band at 69 kD.

For purification of PE II, chromatofocusing was not necessary, and the enzyme extract from CM-cellulose was processed directly on Sephacryl S-200 (Fig. 4), resulting in a pure protein preparation (see SDS-PAGE in Fig. 5a, lane 2).

Mol Wt. The mol wt of PE I was estimated to be 45 kD by gel filtration and 45 to 58 kD by SDS-electrophoresis. The mol wt of PE II determined by gel filtration was 35 to 37 kD, and 40 kD as determined by SDS-PAGE.

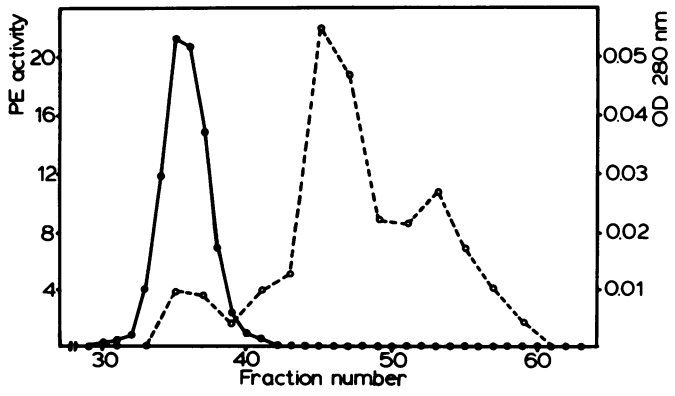

FIG. 3. Gel filtration chromatography on Sephacryl S-200 of the PE I extract after chromatofocusing. The column (1 × 100 cm) was equilibrated and eluted with 50 mM Na-phosphate buffer (pH 6.5). Fractions (1.5 ml) were collected at a flow rate of 2.5 ml/h. (●——●), PE activity (units/30 μl); (○- - -○), $A_{280\ nm}$.

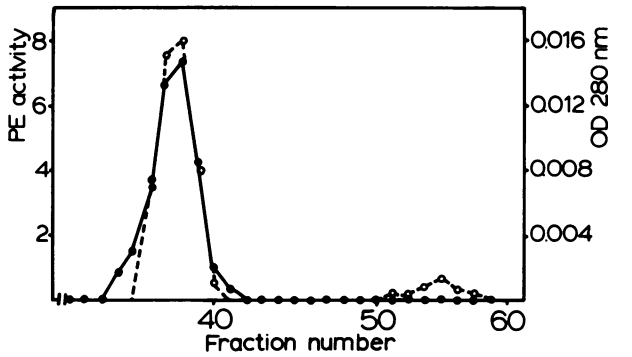

FIG. 4. Gel filtration chromatography on Sephacryl S-200 of the PE II extract after CM-cellulose chromatography. Conditions same as in Figure 3.

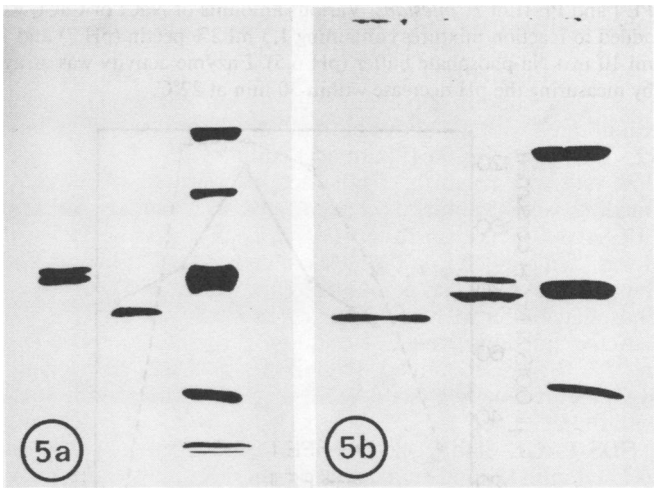

FIG. 5. a, SDS-PAGE of purified extracellular pectinesterases of *P. infestans*. Lane 1, PE I; lane 2, PE II; lane 3, protein mol wt standard with phosphorylase B (92.5 kD), BSA (67kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21.5 kD). b, Glycoprotein assay of purified extracellular pectinesterases of *P. infestans* on SDS-PAGE gels. Lane 1, PE II; lane 2, PE I; lane 3, protein mol wt standard (same as in Figure 5a; phosphorylase B, ovalbumin, and carbonic anhydrase gave a positive reaction).

Further Pectolytic Enzyme Activities of the Purified Enzyme Extracts. Purified PE had no polygalacturonase and pectate lyase activities. PE I showed a very slight pectin lyase activity: in testing an extract with 40 units of PE activity, there was an increase of absorbance of 0.018 within 1 h.

Effect of Salts on PE Activity. The effect of various concentrations of NaCl and $CaCl_2$ on PE activity is illustrated in Figure 6. Both enzymes are stimulated by the addition of salts. PE I was stimulated to a higher degree than PE II. The optimum concentrations were 0.1 M NaCl and 0.01 M $CaCl_2$. NaCl (0.5 M) and 0.1 M $CaCl_2$ were inhibitory to PE II.

PH Optimum of PE Activity. PE I had a good activity at pH 5 to pH 9, the optimum was at pH 7 (Fig. 7). The maximum activity of PE II was within the range between pH 6 and pH 8. There was low activity at pH 4 and no activity at pH 9.

Glycoprotein Assay. Both PEs gave a positive reaction in the modified silver staining procedure on SDS-PAGE gels (Fig. 5b). PE I was adsorbed to Con A-Sepharose, which is another indication for the glycoprotein nature of this protein. The binding was so tight that the protein was not desorbed even by 1 M α-methyl mannoside. For PE II this was not tested.

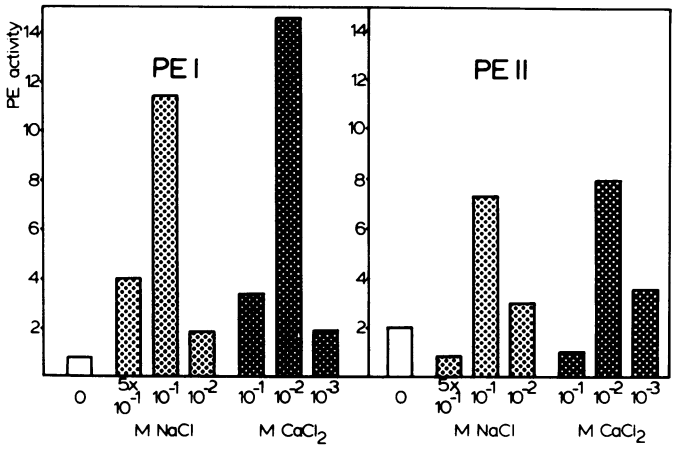

FIG. 6. Effect of NaCl and $CaCl_2$ on activity of purified pectinesterases PE I and PE II of *P. infestans*. Various amounts of NaCl or $CaCl_2$ were added to reaction mixtures containing 1.5 ml 2% pectin (pH 7) and 1.5 ml 10 mM Na-phosphate buffer (pH 6.5). Enzyme activity was assayed by measuring the pH decrease within 30 min at 22°C.

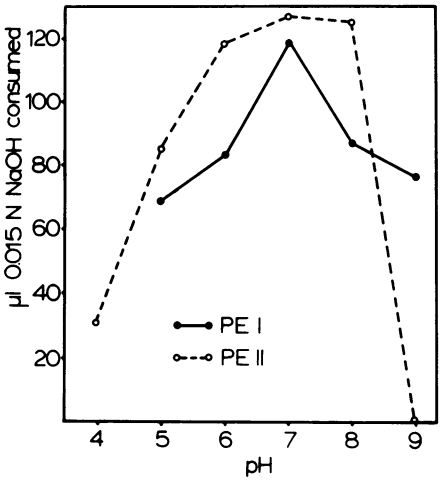

FIG. 7. Effect of pH on PE I and PE II activities. To 9 ml of 2% pectin in 0.25 M NaCl adjusted to pH 4, 5, 6, 7, 8, or 9, 100 µl purified enzyme extract was added. The pH decrease during the reaction was kept constant by adding 0.015 N NaOH. Enzyme activity was expressed by the amount of NaOH consumed within 30 min at 22°C.

DISCUSSION

The ability of pathogens to produce multiple forms of the different polysaccharide-degrading enzymes appears to be common, making the organism more adaptable to changing environmental conditions (9), or these forms might have different tasks. We found that culture filtrate of *P. infestans* contained at least two pectinesterases, PE I and PE II. While PE II was purified to homogeneity, the PE I extract still had two bands in SDS-electrophoresis. Several unsuccessful attempts were made to separate these two proteins: chromatography on hydroxylapatite, different ion exchangers (DEAE-Sepharose and CM-Sepharose), Con A-Sepharose, and cross-linked polypectate (16), hydrophobic interaction chromatography on octyl sepharose, adsorption on bentonit, and chromatofocusing with Pharmacia polybuffer exchanger PBE 118. We suppose that both proteins are PEs. This is substantiated by the facts that, in chromatofocusing of the PE I extract, we always had a double peak in PE activity, and gel filtration did not separate this peak further; but, SDS-PAGE of the single fractions with equal amounts of activity applied showed that the one protein dominated in the first fractions, the other one in the last ones. Thus, assuming that PE I consists of two separate enzymes, we will refer to PE I complex rather than PE I. Jarvis *et al.* (10) found that *P. infestans* produces two PEs, when passing a crude enzyme preparation through a Sephadex G-200 column. No further investigations were made by these authors. Our results show that there are probably three different PEs produced by *P. infestans*, two of them having very similar properties.

In the characterization of both enzyme preparations, we found that the mol wt is about the same size as PEs of other microorganisms (20). The stimulation of PE activity by salts is common, but usually the effect on microbial PEs in contrast to PEs of higher plants is not as great as we found in the case of *P. infestans* enzymes; an increase of 1.5- to 2-fold is reported (20). Since PE activity without adding salts was very low, we assume that the presence of salts is essential for both PEs. The low activity without adding salts might be based on salt impurities in the pectin preparation.

By purifying extracellular PEs of *P. infestans*, we are now able to study their biological significance. In further experiments with specific antibodies, we will try to localize these enzymes in infected plant material and elucidate their role during pathogenesis.

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