Resistance of Gibberellin-Treated Persimmon Fruit to *Alternaria alternata* Arises from the Reduced Ability of the Fungus to Produce Endo-1,4-β-Glucanase

Dani Eshel, Ruth Ben-Arie, Amos Dinoor, and Dov Prusky

First, second, and fourth authors: Department of Postharvest Science of Fresh Produce, Institute for Technology and Storage of Agricultural Products, The Volcani Center, Agricultural Research Organization, P.O. Box 6, Bet Dagan 50250, Israel; and third author: Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agricultural, Food and Environmental Quality Sciences, Rehovot 76100, Israel.
Accepted for publication 7 August 2000.

ABSTRACT

Eshel, D., Ben-Arie, R., Dinoor, A., and Prusky, D. 2000. Resistance of gibberellin-treated persimmon fruit to *Alternaria alternata* arises from the reduced ability of the fungus to produce endo-1,4-β-glucanase. Phytopathology 90:1256-1262.

Black-spot symptoms, caused by *Alternaria alternata*, developed in persimmon fruits during prolonged storage at –1°C. A preharvest treatment with gibberellic acid (GA_3) extended the storage life of the fruit by delaying both black-spot development and fruit softening. Conversely, treatment of persimmon fruits with paclobutrazol (PBZ), an inhibitor of gibberellin (GA) synthesis, enhanced black-spot development and fruit softening during storage. Production of endo-1,4-β-glucanase (EC 3.2.1.4, EG) by *A. alternata* in culture and in the presence of cell walls from PBZ-treated fruits as the carbon source, was enhanced by 150% over production in the presence of cell walls from control fruits, whereas endoglucanase (EG) production in the presence of cell walls from GA_3-treated fruits was reduced by 49% relative to controls. To determine the importance of EG in symptom development, *A. alternata* EG was purified from a culture-inducing medium. It had a molecular mass of 41 kDa, its optimal pH and temperature for activity were 5.5 and 47°C, respectively, and the pI was 4.3. Its K_m and V_{max} were 0.43 mg ml^{-1} and 18 µmol reducing groups minute per milligrams of protein, respectively. The internal sequence of a 21-mer amino acid peptide from the purified EG showed 62% similarity and 38% identity to the EG-1 of *Trichoderma reesei* and of *T. longibrachiatum.* Purified EG induced black-spot symptoms on the fruit, similar to those caused by *A. alternata*, whereas boiled enzyme caused only pricking signs. Our results suggest that the black-spot symptoms caused by *A. alternata*, in persimmon, are related to the ability of the fungus to produce EG in developing lesions.

Additional keywords: postharvest diseases, quiescent infection.

Alternaria alternata (Fr) Keissl. is the causal agent of black spot disease (BSD) in persimmon (*Diospyros kaki* L.) (22). Spores of *A. alternata* land on the fruit's surface and penetrate either directly or via wounds. Direct penetration may occur throughout the growing season and appears to be dependent on the relative humidity (RH) prevailing in the orchard. The infection remains quiescent until the fruit is harvested and only begins to develop slowly during cold storage. Wound penetration may occur through small wounds caused during harvest, but the point of entry is predominantly through small cracks around and beneath the calyx caused by significant fruit growth during the final period of development. Symptoms prior to harvest are observed beneath the calyx under exceptional environmental conditions, when high RH or heavy rains occur in the last month before harvest.

The symptoms after harvest initiate as small black dots and develop into a superficial black, dry rot. The incubation period is long, typically lasting 10 weeks in storage at –1°C. A single treatment of gibberellin acid (GA_3) (50 µg ml^{-1}), applied 10 days before harvest, effectively reduces decay development (20). Perez et al. (20) suggested that the GA_3 effect results from the persistent erect position of the calyx, which reduces localized accumulation of free water on the fruit and consequently prevents fungal development. However, because GA_3-treated fruits were also relatively resistant to the disease following direct inoculation, it was suggested that other factors might also be affecting fungal development in infected fruit. GA_3 inhibits fruit softening during storage (4), whereas paclobutrazol (PBZ), an inhibitor of GA synthesis, induces early fruit ripening and enhanced fruit softening. PBZ-treated fruits also show a higher incidence of decay, thereby shortening their storage potential (6).

Ben Arie et al. (5) suggested that GA_3 treatment increases cellulose content in the cell walls of persimmon fruit. In the present work, we tested the hypothesis that GA_3 treatment affects the production of *A. alternata* cell-wall-degrading enzymes (CWDE), mainly endoglucanase (EG). Our results suggest that the resistance of GA_3-treated persimmons arises from the reduced ability of the fungus to produce an EG capable of digesting the cell walls of the treated fruits during colonization.

MATERIALS AND METHODS

Fruit, fungal isolates, and inoculation procedures. Persimmon fruits (*D. kaki*), showing black spot symptoms, were used for the isolation of *A. alternata*. Following external disinfection of the fruits with 90% ethanol, small slices of peel with black spots were further disinfected with 0.5% NaOCl for 2 min and incubated on potato dextrose agar (PDA) at 25°C. Single-spore cultures were used for fruit inoculation. Conidia were harvested by adding a small amount of sterile distilled water and gently rubbing the sporulating mycelial mat with a bent glass rod. Spore concentration was quantified with a hemacytometer, and 10 µl of the conidial suspension containing 5×10^3 spores was used for fruit inoculation. The fruit was pricked four times at each inoculation site

Corresponding author: D. Prusky; E-mail address: prusky33@netvision.net.il

Publication no. P-2000-0913-01R

with a sterile needle to a depth of 1 to 2 mm. The inoculated fruits were incubated in a moist chamber at 20°C.

Fruit harvesting and assessment of BSD, fruit color, and fruit firmness during storage. For experiments with naturally infected fruits, persimmons were harvested when their color began to change from pale green to light orange. Fruits were usually harvested in a range of colors on the same day and segregated for storage according to their color. Field experiments to evaluate treatment on naturally infected fruits were carried out in a randomized block design with four replications. Each replication was composed of four trees in a row, and fruits were sampled only from the middle two trees. Each field experiment was conducted three times, over two consecutive seasons, in different orchards.

For storage experiments, 30 fruits per replicate were stored at –1°C. After 6 months, the area covered by BSD was estimated by comparison with a diagrammatic scale depicting different areas covered by the disease. The main disease-infected area was around and beneath the calyx. Fruit firmness was evaluated by response to hand pressure, based on an index of 0 (hard) to 4 (very soft). Fruit color was measured with a chromameter (CR-200, Minolta, Osaka, Japan). The three color groups (green, green-orange, and orange) represented three stages of ripening, characterized by chlorophyll degradation and carotenoid biosynthesis (11).

For experiments with artificially inoculated fruits, persimmons were harvested when their color was light orange. Treatments were carried out in a randomized block design with four replications. Each replication was composed of four trees in a row, and fruits were sampled only from the middle two trees. One hundred fruits were harvested from each treatment. Fruits were sorted and 30 were used for inoculation at four points of the upper face of the fruit as already described. The fruits not used for inoculation were used for cell wall preparation (described below). Each field experiment that provided fruit for artificial inoculation was conducted three times, over two consecutive seasons, in the same orchards as those used for naturally infected fruit experiments.

Growth regulators. GA_3 (Fine Agrochemicals, Whittington, England, 4% active ingredient [a.i.]) was applied in the orchard with a hand-operated spray gun at a pressure of 20 kg cm^{-2}. One spray of GA_3 at concentrations of 50, 100, or 200 μg ml^{-1} was applied, and each tree was sprayed with 7 to 8 liters of spray mix. Treatments were applied 10 days before harvest in three orchards in the coastal plain of Israel, where BSD is most prevalent. Two liters of 250 μg ml^{-1} of PBZ (Cultar, Machteshim, Israel, 25%, wt/vol a.i.), was applied to the soil around each tree at fruit set.

Persimmon cell wall preparation. Cell walls were prepared, in principle, according to Harborne (12). Flesh (mesocarp; 300 g) was ground in 350 ml of acid-methanol (4% HCl, vol/vol) and filtered through Whatman No. 1 filter paper (Whatman, Maidstone, England). The residue was collected from the filter paper and blended again twice in a similar amount of methanol until no phenols could be detected in the filtrate. Phenols were checked by adding 1 ml of a 5% solution of $FeCl_3$ (Sigma Chemical Co., St. Louis) to 4-ml samples of the filtrate; black color indicated their presence (9). The residue was blended with 200 ml of cold acetone three times and air-dried overnight before weighing. The air-dried residue was kept at –20°C until used as a carbon source in the liquid media.

Extraction of EG from liquid culture. *A. alternata* was grown in 1-liter flasks, each containing 400 ml of EG-inducing medium (EIM). The EIM was based on the medium described by Mandels and Weber (18) and contained (gram $liter^{-1}$ in distilled water): Avicel (Fluka, Neu-Ulm, Switzerland), 10; proteose peptone (Difco Laboratories, Detroit), 0.5; Tween 80, 0.2; urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2$, 0.3; $FeSO_4$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.0014; $CoCl_2$, 0.002; $MnSO_4$, 0.0016. The medium was autoclaved at 121°C for 15 min, and the pH was adjusted to 3.8 with 1 M H_3PO_4.

A. alternata was also grown using a cell wall preparation of untreated, GA- and PBZ-treated persimmon fruits. The cell wall preparation was added to the EIM instead of the Avicel substrate as an inducing substrate. Each flask was inoculated with 4×10^7 spores and incubated on an orbital shaker (160 rpm) at 22°C for 10 days. The effect of different cell wall source or Avicel on fungal growth rate of *Alternaria* was determined first by vacuum-filtering through a glass microfiber filter (GF/C, Whatman) and storing the mycelia at 105°C for 48 h and weighing the mycelia.

Purification of EG and characterization. The culture medium was vacuum-filtered through a glass microfiber filter concentrated by lyophilization, and the powder was dissolved in 400 ml of distilled water and differentially precipitated with 90% ammonium sulfate. The ammonium sulfate-precipitated protein was dissolved in 20 ml of 0.2 M acetate buffer (pH 3.5) and dialyzed extensively at 4°C, against three changes of 5 liters of the same buffer every 4 h. The dialyzed solution was concentrated by Centriprep 10 (Amicon, Beverly, MA) to a volume of 10 ml and applied to a column (15 × 3 cm) of Amberlite CG-50 (Aldrich, Milwaukee, WI), previously equilibrated with the same acetate buffer as that used for dialysis. The enzyme was eluted stepwise with three column volumes of three concentrations of acetate buffer (first step = 0.2 M, pH 3.5; second step = 0.3 M, pH 4.5; and third step = 0.4 M, pH 5.5) and 10-ml fractions were collected. EG-active fractions, eluted with 0.4 M acetate buffer, were desalted by dialysis against water and concentrated by Centriprep 10. To further purify the active fraction, the dialyzed solution was subjected to preparative isoelectric focusing (IEF) by a column with 1% carrier ampholytes (LKB 8100-1, Bio-Rad Laboratories, Hercules, CA). The sample was applied to a 50 to 5% (wt/vol) linear sucrose gradient and electrophoresed at a constant power of 7 W at 4°C and a final 2,000 to 2,500 V for 16 h. The pH, absorbance at 280 nm, and enzyme activity were measured in 2-ml fractions. The active fractions were pooled and dialyzed against 0.05 M acetate buffer (pH 5.5) and subsequently the volume was adjusted to 4.5 ml.

To determine the optimal temperature, enzyme activities were measured as described earlier during 1-h exposure to various temperatures. To assess thermal stability, the enzymes were treated at various temperatures for 30 min at pH 5.5 without substrate, after which activities were measured. Optimal pH was determined in the presence of acetate buffer at pH 3.5 to 7.5 and Tris-HCl buffer at pH values above 7.5. The K_m and V_{max} of the purified enzyme were calculated from a Lineweaver-Burk plot, based on the reducing-group assays.

EG assays. EG activity was assayed spectrophotometrically by measuring the release of reducing sugar from sodium carboxymethylcellulose (CMC; Sigma). The method was based on that of Somogyi (26) and Nelson (19). The reaction mixture contained 0.3 ml of 0.5% CMC dissolved in 0.1 M acetate buffer (pH 5.5) and 0.1 ml of enzyme solution. At different periods during incubation at 37°C, the reaction was stopped and the product was analyzed by measuring the optical density at 660 nm. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 0.5% CMC to produce reducing sugar equivalent to 1 μmol D-glucose per minute under standard assay conditions. Specific activity was expressed as units per milligram of protein as measured by a dye-binding assay (Bio-Rad), with crystalline bovine serum albumin as the reference standard.

To localize the enzyme in different fractions during its purification, EG was also assayed by the cup-plate diffusion assay (16). Plates were prepared by dissolving 0.5% CMC and 1.5% agar in 0.1 M citrate phosphate buffer (pH 5.5), under constant stirring and heating. A siliconized cup-former (5-mm diameter) was dipped in the agar medium, creating cups with volumes of ≈20 μl. These cups were filled with 20 μl of the test solutions and incubated for ≈16 h at 37°C. For visualization of hydrolysis, the plates were first flooded with a 0.1% aqueous solution of Congo red (Sigma) for at least 30 min, and an additional 10-min rinse with 1 M NaCl.

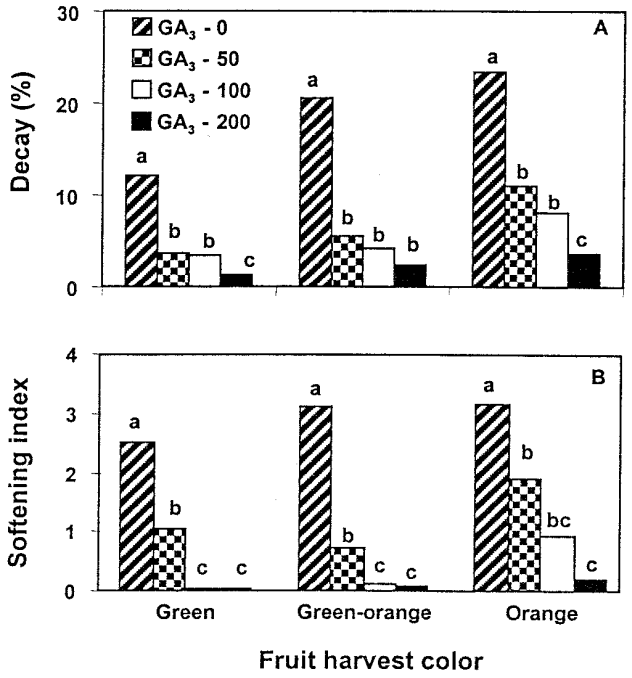

Fig. 1. Effect of preharvest gibberellic acid treatments (GA_3, micrograms per milliliter) on natural incidence of *Alternaria alternata* symptoms and fruit firmness in persimmon fruits harvested at ripening (color) stages, after 6 months storage at –1°C. **A,** Decayed area of the fruit. **B,** Fruit softening index of 0 (hard) to 4 (very soft). Trees were sprayed with GA_3 10 days before harvest. Each point represents the average of 120 fruits (30 fruit × 4 replications). The effect of GA_3 concentration on symptom development was analyzed separately for each maturity stage (harvesting color). Within each harvesting color, average values indicated by different letters differ significantly ($P \leq 0.05$).

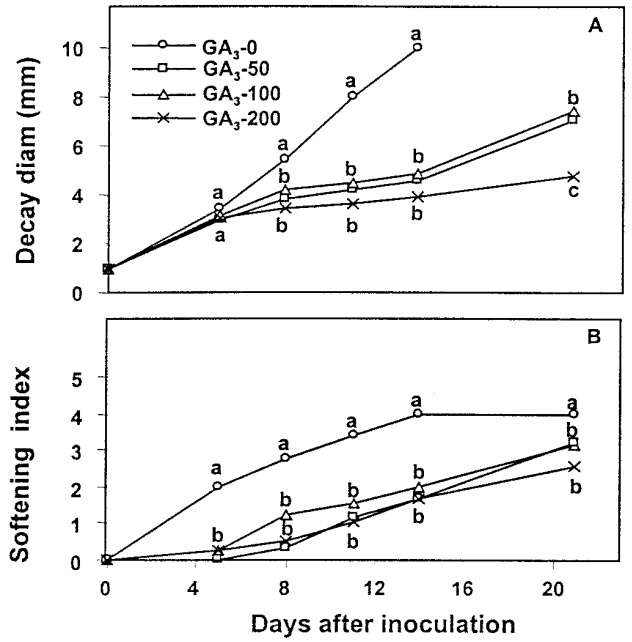

Fig. 2. Effect of preharvest gibberellic acid treatments (GA_3, micrograms per milliliter) on postharvest *Alternaria alternata* symptom development and fruit firmness in inoculated persimmon fruits incubated at 20°C. **A,** Decay diameter at inoculation site. **B,** Fruit softening index of 0 (hard) to 4 (very soft). The trees were sprayed with GA_3 10 days before harvest. Each point represents the average of 480 inoculations (30 fruit × 4 inoculation points × 4 replications). Average values on each day after inoculation indicated by the same letters are not significantly different according to one-way analysis of variance at $P \leq 0.05$.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, IEF gel, and activity gel. Two parts purified enzyme was mixed with one part loading buffer (150 mM Tris-HCl (pH 6.8); 2% sodium dodecyl sulfate [SDS]; 3% Bromophenol blue [Sigma]; and 30% glycerol) and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) using a 10% acrylamide resolving gel (25). The gel was divided and one half was stained to detect protein with 0.1% Coomassie brilliant blue R-250 in 10% acetate and 50% methanol for less than an hour. The other half was used to detect activity by incubating the gel in a plastic plate over a 1- to 2-mm layer of 0.5% CMC and 1.5% agar in 0.1 M citrate phosphate buffer (pH 5.5) for ≈30 min at 47°C. For visualization of substrate hydrolysis, the plate was first flooded with a 0.1% aqueous solution of Congo red for at least 30 min, and an additional 10-min rinse with 1 M NaCl.

The molecular mass of EG was estimated by comparison with Kaleidoscope Prestained Standards (Bio-Rad) containing: myosin, 200 kDa; β-galactosidase, 118 kDa; bovine serum albumin, 75 kDa; carbonic anhydrase, 43 kDa; soybean trypsin inhibitor, 32.1 kDa; lysozyme, 17.8 kDa; and aprotinin, 7.2 kDa.

Crude or purified enzyme preparations were subjected to IEF using vertical Pre-Cast IEF gels (Novex, San Diego) in a pH range of 3 to 10. The gel was divided and half was fixed in 3.5% (wt/vol) sulfosalicylic acid and 11.5% (wt/vol) trichloroacetic acid (Sigma) in water for 30 min and then stained as described previously. The other half of the gel was used to detect activity (described below). The pI was estimated by IEF markers 3 to 10 (Serva, Heidelberg, Germany) that contained: cytochrome c, pI 10.7; ribonuclease A, pI 9.5; lectins, pI 8.3, 8.0, and 7.8; myoglobins, pI 7.4 and 6.9; carbonic anhydrase, pI 6.0; β-lactoglobu-

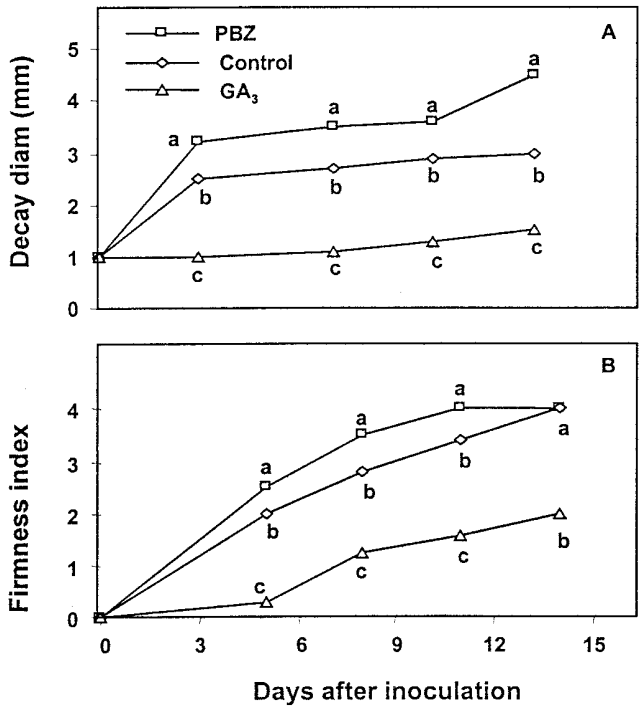

Fig. 3. Effect of orchard application of growth regulators on postharvest development of *Alternaria alternata* symptoms and fruit firmness in inoculated persimmon fruits incubated at 25°C. **A,** Decay diameter at inoculation site. **B,** Fruit softening index of 0 (hard) to 4 (very soft). Paclobutrazol (PBZ) was applied at fruit set to the soil around each tree. Gibberellic acid (GA_3; 50 μg ml^{-1}) was applied 10 days before harvest. After harvest, fruits were prick-inoculated as described in Material and Methods. Each point represents the average of 480 inoculations (30 fruit × 4 inoculation points × 4 replications). Average values on each day after inoculation indicated by the same letters are not significantly different according to one-way analysis of variance at $P \leq 0.05$.

lins, pI 5.3 and 5.2; trypsin inhibitor, pI 4.5; glucose oxidase, pI 4.2; and amyloglucosidase, pI 3.5.

Protein sequence determination. The purified EG was subjected to SDS-PAGE and, following staining, the protein bands were excised and sequenced at the Protein Research Center, Department of Biology, Technion, Haifa, Israel. Sequences were analyzed at the SWISS-PROT databank.

Treatment of persimmon fruits with purified EG. Fruits were disinfected with 90% ethanol, left to dry, and pricked three times at each inoculation site with a sterile needle to a depth of 1 to 2 mm. Enzyme solutions (5 U in 10 µl of H_2O) were placed on the wounded site and, for comparison, a 30-min boiled enzyme solution was used as a control. Following enzyme treatment, the fruits were covered with polyethylene bags to maintain high humidity and held at 26°C for 2 days.

Statistical analyses. Each field experiment was conducted three times. Separate analysis of each experiment showed homogeneous variances of the experimental error between the repeats. Analysis of the data showed no significant interaction between the treatments and the replicate experiments; only the effect of the treatments (e.g., decay area and softening) is shown in the analyses. The percentage of decay area was arcsine-transformed before analysis. The effect of growth regulators was tested by one-way analysis of variance. All analyses were performed with the SAS program (SAS Institute, Cary, NC, release 6.04 for PC) at $P \leq 0.05$.

RESULTS

Effect of GA_3 on BSD incidence and firmness of naturally infected fruits during storage. The area of decay on naturally infected fruits after 6 months of storage at –1°C increased from 12 to 23% with the change in fruit color at harvest from green to orange (Fig. 1A). The decayed area was significantly reduced at each color stage when fruits were sprayed with 50 µg ml^{-1} of GA_3 prior to harvest, and the extent of that reduction increased with increasing GA_3 concentration to 200 µg ml^{-1}. GA_3 treatment at concentrations of 100 and 200 µg ml^{-1} prevented fruit softening almost completely during storage (Fig. 1B). For orange fruits, only the highest GA_3 treatment prevented loss of fruit firmness, although treatment with 50 and 100 µg ml^{-1} significantly reduced softening.

Effect of GA_3 and PBZ on BSD and firmness of inoculated fruits. GA_3-treated fruits harvested at the orange stage, which were prick-inoculated immediately after harvest and stored at 20°C, exhibited a significant reduction in decay diameter and firmness loss relative to untreated fruits (Fig. 2). The highest dose, 200 µg ml^{-1} did not reduce decay and fruit softening more significantly than 50 or 100 µg ml^{-1}, for most measurements (Fig. 2). No effect on in vitro fungal development was observed when *A. alternata* was grown on PDA in the presence of GA_3 at concentrations of up to 200 µg ml^{-1} (data not shown).

Preharvest treatments with PBZ enhanced BSD development in harvested fruits by 30% in fruit that had been prick-inoculated,

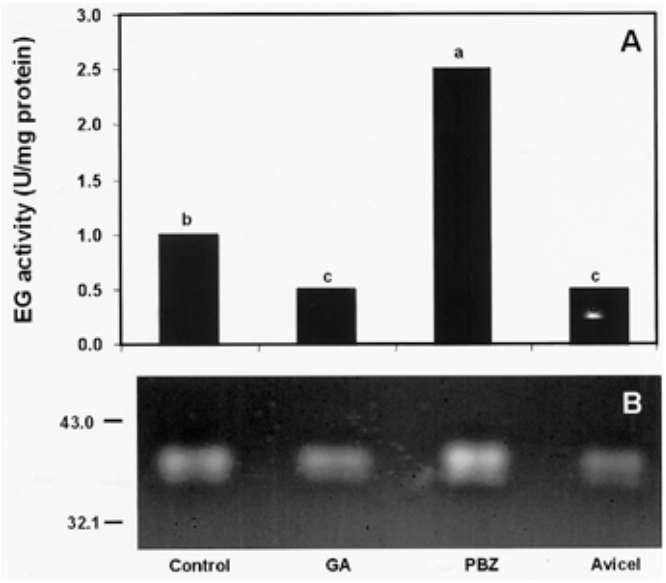

Fig. 4. A, Activity assay and **B,** Carboxymethylcellulose (CMC) activity gel of culture filtrate of endoglucanase (EG) produced by *Alternaria alternata* in the presence of Avicel, persimmon cell walls extracted from untreated (Control) and growth-regulators-treated fruits. Cell walls or Avicel were added to the liquid media (1%, wt/vol) as described in Material and Methods. Avicel was used as a positive control. Activity assay values represent the average EG activities present in the supernatant of five different flasks 10 days after inoculation. Experiments were repeated three times and the results presented were obtained from one experiment. Similar results were obtained in the other experiments. Average values indicated by the same letters are not significantly different according to one-way analysis of variance at $P \leq 0.05$. Activity gel of the concentrated culture filtrate: 5 µg of nonboiled total protein per lane was electrophoresed at pH 8.8 on a 10% acrylamide gel. The gel was blotted onto an agar-CMC plate for 30 min at 47°C. For visualization of hydrolysis, the plates were flooded with a 0.1% aqueous solution of Congo red for at least 30 min followed by an additional 10-min rinse with 1 M NaCl.

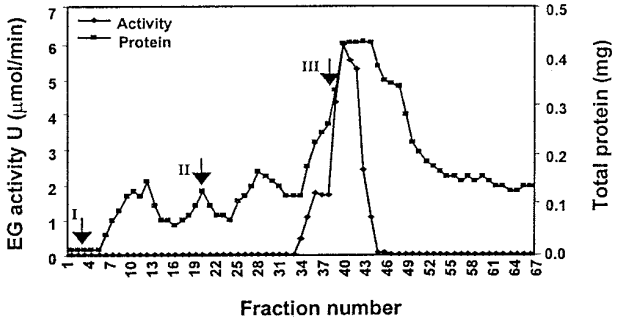

Fig. 5. Elution profile of protein and endoglucanase activity on an Amberlite CG-50 ion-exchange column. The column was eluted stepwise with three acetate buffers: I = 0.2 M, pH 3.5; II = 0.3 M, pH 4.5; and III = 0.4 M, pH 5.5.

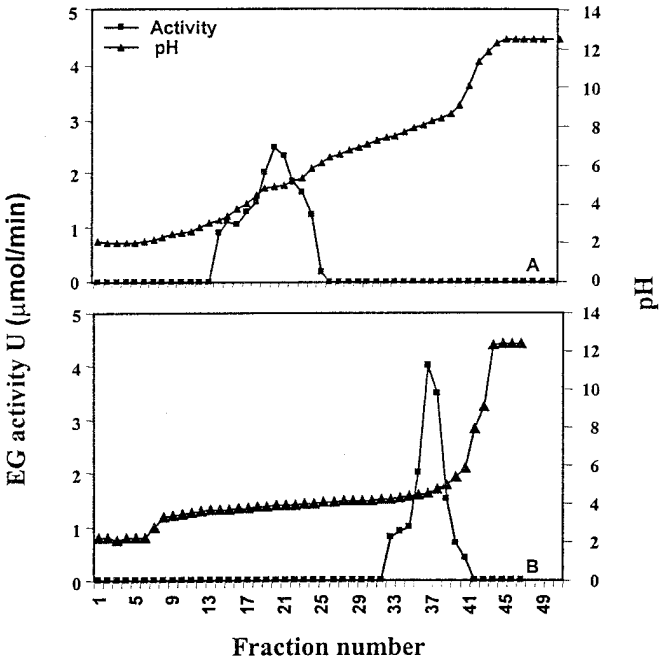

Fig. 6. Isoelectric focusing (IEF) of the active fraction collected from the Amberlite ion-exchange column. **A,** IEF using ampholines in a pH range from 3 to 10. **B,** IEF of the active fraction collected from **A** with a narrower range of ampholines (pH 3 to 5).

TABLE 1. Purification of endoglucanase (EG) from culture filtrate of *Alternaria alternata* persimmon pathotype

Treatment[a]	Volume (ml)	Total protein (mg)	Total EG (unit)	Specific activity (unit mg^{-1})	Yield (%)	Purification factor (fold)
Ammonium sulfate (90%)	50	24.2	38.0	1.6	100	
Amberlite CG-50	130	3.3	31.8	7.3	83.7	4.7
First IEF pH 3–10	24	0.5	16.5	34.2	43.3	21.8
Second IEF pH 3–5	18	0.3[b]	14.8	54.8	38.9	34.9

[a] IEF = isoelectric focusing.
[b] The protein content of the final EG fraction isolated from 12 liters of culture filtrate was 0.3 mg with a specific activity of 54.8 unit mg^{-1}.

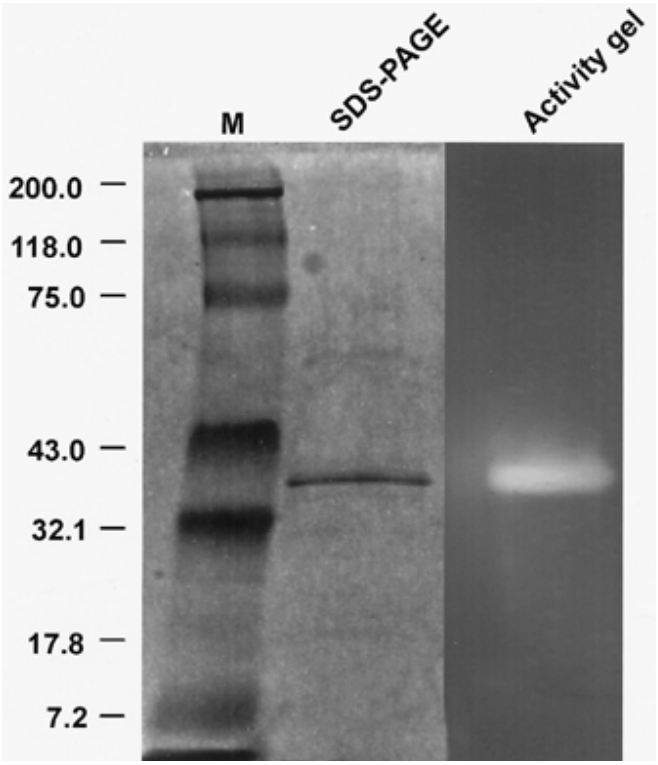

Fig. 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and carboxymethylcellulose (CMC) activity gel of the purified endoglucanase from *Alternaria alternata*. The enzyme (10 µg for protein detection, and 0.5 µg for activity gel) was electrophoresed at pH 8.8 on a 10% acrylamide gel. One half of the gel was stained with Coomassie brilliant blue R-250. The other half was blotted on an agar-CMC plate for 30 min at 47°C. For visualization of hydrolysis, the plates were flooded with a 0.1% aqueous solution of Congo red for at least 30 min, followed by an additional 10-min rinse with 1 M NaCl.

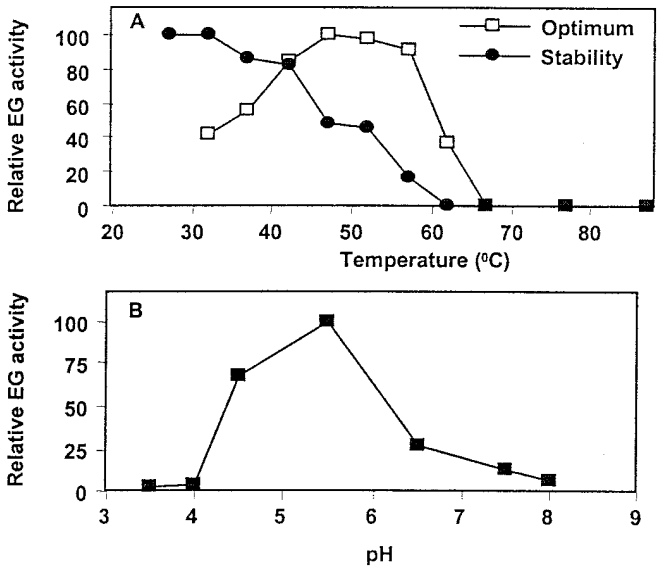

Fig. 8. Effect of temperature and pH on purified endoglucanase activity. **A,** Assays conducted during exposure to temperature (optimum) following 30-min exposure (stability). **B,** Effect of pH.

whereas lesion areas on GA_3-treated fruits were reduced by 60% relative compared with untreated fruits (Fig. 3A). Treatment with PBZ enhanced loss of fruit firmness in comparison with untreated and GA_3-treated fruits (Fig. 3B).

EG production by *A. alternata* grown on Avicel and cell walls of persimmon. *A. alternata* secreted EG when grown on EIM containing Avicel as the sole carbon source. However, persimmon cell walls were a better inducer of EG production than Avicel (Fig. 4A). When *A. alternata* was grown on EIM containing cell walls from PBZ-treated fruits, EG production was enhanced by 150% over the control fruits. With cell walls from GA_3-treated fruits, EG production was reduced by 49% relative to the control (Fig. 4A). EG activity in EIM containing fruit cell walls was the result of one single band with the same molecular mass (41 kDa) as the EG produced in EIM containing Avicel (Fig. 4B). EG activity and hyphae mass produced by *A. alternata* in EIM containing Avicel amended with up to 200 µg ml^{-1} of GA_3 was not affected (data not shown).

Purification and characterization of EG. Maximum EG activity in EIM containing Avicel was reached ≈16 days after inoculation (data not shown). The pH of the culture filtrate at 16 days was 6.4. Most of the EG activity in the culture filtrate precipitated out in 90% ammonium sulfate (Table 1). The EG eluted from Amberlite (Fig. 5) was first purified by IEF in pH range 3 to 10 (Fig. 6A), and the active fractions further subjected to a second IEF column, with a narrower range of ampholines (pH 3 to 5) (Fig. 6B). Finally, EG was purified 35-fold with a 39% yield (Table 1).

The purified EG, produced in the presence of Avicel, was analyzed by 10% SDS-PAGE and estimated to have a molecular mass of 41 kDa (Fig. 7). The pI determined by IEF column was approximately 4.3 (Fig. 6A and B), and this value was also confirmed by IEF activity gel (data not shown). The optimum pH for activity was 5.5 (Fig. 8B), the optimum temperature was 47°C, and thermal stability was completely lost at 62°C (Fig. 8A). The *A. alternata* EG showed Michaelis-Menten kinetics for CMC as a substrate. The K_m value of the isolated EG was approximately 0.43 mg ml^{-1} of CMC, and its V_{max} was 1.8×10^{-2} mmol reducing groups minute per milligram of protein.

The purified EG was subjected to trypsin digestion. A 21-mer amino acid peptide had the following sequence: Ile-Gly-Pro-Glu-Ile-Phe-Ser-Val-Asp-Thr-Ser-Ala-Val-Pro-Ala-X-Gln-Thr-Gly-Phe-Phe. This sequence was compared by SWISS-PROT database and exhibited 62% similarity and 38% identity to EG-1 of *Trichoderma reesei* (SWISS-PROT accession no. Q12714) and *T. longibrachiatum* (P07981).

Effect of purified EG from *A. alternata* on symptom formation in persimmon fruit. Purified EG placed on pricked fruit mimicked the development of black spot symptoms developed on infected fruit within 2 days (Fig. 9), whereas boiled enzyme only produced pricking signs.

DISCUSSION

The most noticeable effect of preharvest GA_3 treatment on stored persimmon fruits was the prevention of BSD caused by *A. alternata*. Maximal inhibition was obtained with a single treat-

ment of 200 μg ml^{-1} of GA_3 10 days before harvest, although with most of the measurements there was no significant difference in 50, 100, and 200 μg ml^{-1} of GA_3. Naturally infected fruits showed a significant increase in the percentage of decay as the fruit color changed from green to orange, in both untreated fruits and at all GA_3 doses tested, suggesting that fruit susceptibility to disease increases with ripening. This is similar to reports obtained from other fruits such as apricot, banana, mango, and avocado (21). However, GA_3-treated orange persimmon fruits naturally infested or prick-inoculated with *A. alternata* exhibited the same reduced susceptibility to BSD as green, untreated fruits, suggesting that GA_3 does not prevent decay development simply by affecting fruit maturity, as reflected by color. To determine the mode of action of GA_3 on BSD, we first examined whether resistance in GA_3-treated fruits is determined by antifungal compounds. However, no correlation was detected between the concentration of *A. alternata* inhibitors in fruit tissue (peel or flesh) and GA_3 treatment (D. Eshel, R. Ben-Arie, A. Dinoor, and D. Prusky, *unpublished data*).

There is some evidence that the cell wall is one of the sites responsive to GA_3's effects in ripening fruit (5,13,17) and vegetative tissue (14). GA_3 delayed fruit softening at all stages of ripening and the same trend was maintained in wounded and inoculated persimmon fruits (4,15). Because cellulose microfibrils provide the structure and support for all the components of plant cell walls (7,10), the increase in cellulose would increase fruit firmness. Ben-Arie et al. (5) suggested that the greater firmness of GA_3-treated fruits accounts for the 37% higher cellulose content in the cell walls of the treated fruit. At the same time, Ben Arie et al. (5) also found that fruit EG activity at harvest is very low in control fruits and was undetectable in GA_3-treated fruits (5). Both factors, cellulose synthesis and hydrolytic activity, probably affect fruit firmness as determined by GA_3 treatment. Because PBZ inhibits endogenous GA synthesis (23,27,29), it is not surprising that treatment with PBZ at fruit set increased *A. alternata* symptom development. Increased susceptibility may be the result of cell wall weakening caused by a reduction in the amount or density of cellulose and this could lead to enhanced degradation of cell walls by fungal glucanases or CWDE (8).

There is very little evidence supporting the importance of EG among the CWDE secreted by *A. alternata* (3). This is the first report showing the relationship among EG production, BSD, and the effect of GA_3 on resistance of persimmon fruits. The first step in testing this relationship was to purify and characterize *A. alternata* EG and to show its involvement in BSD symptom formation. When grown on commercial cellulose or persimmon cell walls, *A. alternata* produced a single active EG with a molecular mass of 41 kDa, resembling that of *Fusarium* and *Trichoderma* EGs (28). The pH optimum of the purified EG is 5.5, which is compatible with other fungal EGs whose values range from 4.0 to 5.5. The pI value of 4.3 is close to that of the *Trichoderma* EGs (28). When the purified enzyme was placed on the pricked persimmon tissue, it mimicked the development of black-spot symptoms caused by the pathogen. It is still not clear how EG affects black-spot development. It may be that EG causes cell necrosis as a result of cellulose digestion that in turn activates an oxidative reaction (e.g., an increase in polyphenol oxidase activity [1,2]), or it might indirectly elicit programmed cell death (24).

A second approach to determining the relationship between EG production and *A. alternata* attack was to grow the fungus in the presence of cell walls from resistant and susceptible fruits. EG production was enhanced by 150% over control fruits when *A. alternata* was grown on cell walls of PBZ-treated susceptible fruits. In contrast, EG production in the presence of cell walls from GA_3-treated resistant fruits was reduced by 49% relative to controls. The different levels of EG activity reflect the secretion of a single EG of 41 kDa, suggesting that the different substrates have an affect on the amount of EG production and not the appearance of new EG isozymes. Because GA_3 added to the growth medium did not affect EG production in vitro when *A. alternata* was grown in the presence of different substrates, we can assume that GA_3 has no direct effect on the fungus and that its effect is indirect, mainly on the cell-wall structure of the host.

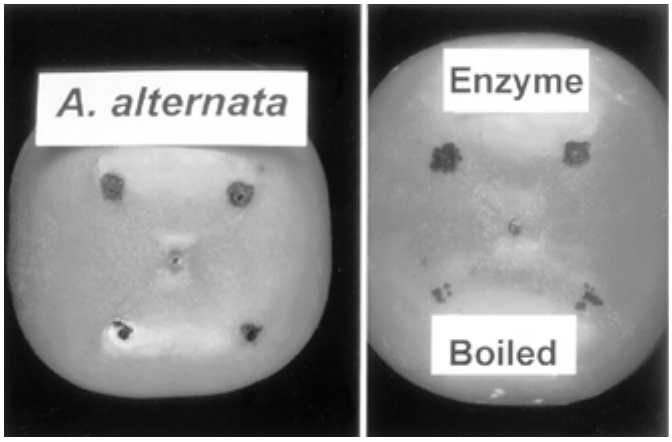

Fig. 9. Effect of purified endoglucanase (EG) from *Alternaria alternata* on persimmon fruit. Five units of the purified EG in 10 μl of water were prick inoculated on the fruit and incubated at 26°C for 2 days (enzyme). The same amount of enzyme was boiled for 30 min and used as a control (boiled).

The lesions mimicking black-spot symptoms elicited by the purified enzyme further indicate EG's involvement in symptom development caused by *A. alternata*. This traditional approach is, however, not conclusive evidence for the involvement of EG in virulence. Expression of EG's in the infected tissue attacked by *A. alternata* has been found (D. Eshel, R. Ben-Arie, A. Dinoor, and D. Prusky, *unpublished data*). However, conclusive experiments demonstrating the role of EG in symptom development will be obtainable only when the gene is cloned and disrupted.

LITERATURE CITED

1. Ahl-Goy, P., Felix, G., Metraux, J. P., and Meins, F., Jr. 1992. Resistance to disease in the hybrid *Nicotiana glutinosa* × *Nicotiana debneyi* is associated with high constitutive levels of β-1,3-glucanase, chitinase, peroxidase, and polyphenol oxidase. Physiol. Mol. Plant Pathol. 41:11-21.
2. Avdiushko, S. A., Ye, X. S., and Kuc, J. 1993. Detection of several enzymatic activities in leaf prints of cucumber plants. Physiol. Mol. Plant Pathol. 42:441-454.
3. Barkai-Golan, R., and Kopeliovitch, E. 1989. Effect of peel injury and enzymatic activity of the fruit on the tolerance of tomato genotypes to *Alternaria* infection. Acta Hortic. 258:631-637.
4. Ben-Arie, R., Bazak, H., and Blumenfeld, A. 1986. Gibberellin delays harvest and prolongs storage life of persimmon fruits. Acta Hortic. 179:807-813.
5. Ben-Arie, R., Saks, Y., Sonego, L., and Frank, A. 1996. Cell wall metabolism in gibberellin-treated persimmon fruits. Plant Growth Regul. 19:25-33.
6. Ben-Arie, R., Zhou, H. W., Sonego, L., and Zutkhi, Y. 1997. Plant growth regulator effects on the storage and shelf-life of 'Triumph' persimmons. Acta Hortic. 436:243-250.
7. Carpita, N. C., and Gibeaut, D. M. 1993. Structural models of primary cell walls in flowering plant: Consistency of molecular structure with the physical properties of the walls during growth. Plant J. 3:1-30.
8. Cooper, R. M. 1983. The mechanisms and significance of enzymatic degradation of host cell walls by parasites. Pages 101-137 in: Biochemical Plant Pathology. J. A. Callow, ed. Wiley Press, Chichester.
9. Gazit, S., and Levy, Y. 1963. Astringency and its removal in persimmon. Israel J. Agric. Res. 13:125-132.
10. Greve, L. C., and Labavitch, J. M. 1991. Cell wall metabolism in ripening fruit. V. Analysis of cell wall synthesis in ripening tomato pericarp tissue using a D-[U-^{13}C]-glucose tracer and gas-chromatography-mass spectrometry. Plant Physiol. 97:1455-1461.
11. Gross, J., Bazak, H., Blumenfeld, A., and Ben-Arie, R. 1984. Changes in chlorophyll and carotenoid pigments in the peel of 'Triumph' persimmon (*Diospyros kaki* L.) induced by pre-harvest gibberellin (GA_3) treatment. Sci. Hortic. 24:305-314.
12. Harborne, J. B. 1958. Spectral methods of characterizing anthocyanins. Biochem. J. 70:22-28.

13. Jona, R., Goren, R., and Marmora, M. 1989. Effect of gibberellin on cell wall components of creasing peel in mature Valencia orange. Sci. Hortic. 39:105-115.
14. Jones, R. L. 1982. The role of gibberellins in plant cell elongation. Crit. Rev. Plant Sci. 1:23-47.
15. Kang, I. K., Suh, S. G., Gross, K. C., and Byun, J. K. 1994. N-terminal amino acid sequence of persimmon fruit β-galactosidase. Plant Physiol. 105:975-979.
16. Kollar, A. 1994. Characterization of specific induction, activity, and isozyme polymorphism of extracellular cellulases from *Venturia inaequalis* detected in vitro and on the host plant. Mol. Plant-Microbe Interact. 7:603-611.
17. Lewis, L. N., Coggins, C. W., Labanauskas, C. K., and Dugger, W. M. 1967. Biochemical changes associated with natural and gibberellin A_3 delayed senescence in the Navel orange rind. Plant Cell Physiol. 8:151-160.
18. Mandels, M., and Weber, J. 1969. The production of cellulases. Pages 391-413 in: Cellulases and Their Applications, Advances in Chemistry Series 95. E. J. Hajny and E. T. Reese, eds. American Chemical Society, Washington DC.
19. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.
20. Perez, A., Ben-Arie, R., Dinoor, A., Genizi, A., and Prusky, D. 1995. Prevention of black spot disease in persimmon fruit by gibberellic acid and iprodione treatments. Phytopathology 85:221-225.
21. Prusky, D. 1996. Pathogen quiescence in postharvest diseases. Annu. Rev. Phytopathol. 34:413-434.
22. Prusky, D., Ben-Arie, R., and Guelfat-Reich, S. 1981. Etiology of black spot disease caused by *Alternaria alternata* in persimmons. Phytopathology 71:1124-1128.
23. Rademacher, R. 1990. New types of plant growth retardants: Additional perspectives for practical applications in agriculture and horticulture. Pages 611-618 in: Plant Growth Substances. R. P. Pharis and S. B. Rood, eds. Springer-Verlag, Berlin.
24. Ryerson, D. E., and Heath, M. C. 1996. Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell 8:393-402.
25. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. Somogyi, M. 1952. Notes on sugar determination. J. Biol. Chem. 195:19-23.
27. Talon, M., Zacarias, L., and Primo-Millo, E. 1992. Gibberellins and parthenocarpic ability in developing ovaries of seedless mandarins. Plant Physiol. 99:1575-1581.
28. Wood, T. M. 1991. Fungal cellulases. Pages 491-535 in: Biosynthesis and Biodegradation of Cellulose. C. H. Haigler and P. J. Weimer, eds. Marcel Dekker, Inc., New York.
29. Zacarias, L., Talon, M., Ben-Cheikh, W., Lafuente, M. T., and Primo-Millo, E. 1995. Abscisic acid increases in non-growing and paclobutrazol-treated fruits of seedless mandarins. Physiol. Plant 95:613-619.