Relationship of Phytophthora Fruit Rot to Fruit Maturation and Cuticle Thickness of New Mexican-type Peppers

Charles L. Biles, Marisa M. Wall, Mark Waugh, and Heather Palmer

First, third, and fourth authors: College assistant professor, research specialist, and research assistant, Department of Entomology, Plant Pathology and Weed Science; and second author: Assistant professor, Department of Agronomy and Horticulture, New Mexico State University, Las Cruces 88003.

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ABSTRACT

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Experiments were conducted to determine the relationship between pepper fruit susceptibility to Phytophthora fruit rot and fruit maturity. Pepper fruits were harvested weekly throughout the 1991 growing season and were dipped in a suspension of *Phytophthora capsici* zoospores (5×10^3 zoospores/ml). The susceptibility of nonwounded fruit to infection decreased with increased ripening. Lesion elongation in nonwounded fruit decreased from 14.1 mm/day in green fruit to 10.7 mm/day in nondehydrated red fruit. Nonwounded fruit (immature green, mature green, and red) inoculated with 5×10^3 zoospores in a 100-μl drop also exhibited less infection as the fruit ripened. Rates of lesion elongation were 9.6 mm/day, 5.9 mm/day, or 2.7 mm/day in nonwounded immature green, mature green, or red fruit, respectively. In contrast, lesion growth rate in wounded fruit was 14.0 mm/day for all maturation stages. Fungal growth was greater in red fruit extracts than in green fruit extracts, which suggested that inhibitory mechanisms, such as fungal chemical inhibitors, were not present in red fruit. Fungal growth was directly related to the sugar content of the fruit. Cuticle thickness, however, increased from 12 μm in immature green fruit to 24 μm in red fruit. Peroxidase activity and isoforms increased with ripening. Nitrocellulose tissue blotting indicated increased peroxidase activity in the cuticular region as the fruit matured, which suggested increased polymerization of ligninlike compounds and an increased barrier to infection. Cuticle thickness of nonwounded mature green and red pepper fruit appears to be a factor in resistance to *P. capsici*.

Additional keywords: *Capsicum annuum*, isozymes.

The New Mexican-type pepper (*Capsicum annuum* L. long, green type) is widely grown in the southwestern United States. Mature green peppers, called chile, are consumed fresh or processed for canning, and red fruits are dehydrated for use as chile powder or paprika. In New Mexico, Phytophthora blight of pepper occurs on the roots, stems, leaves, and fruits (3,14,22) and severely reduces yield and fruit quality. The pathogen, *Phytophthora capsici* Leonian, is also pathogenic on tomato, eggplant, cucumber, watermelon, pumpkin, squash, cocoa, and macadamia (10,11,13,18,21).

On peppers, Phytophthora fruit rot may infect fruits during prolonged periods of heavy rainfall and high humidity, especially when plants are overcrowded or overfertilized with nitrogen (22). Infected fruits initially show water-soaked lesions and eventually shrivel and rot. The fruits turn white, and the interior of the pods are heavily colonized by the white mycelium of *P. capsici* (22).

Although research on Phytophthora root rot of peppers and breeding for resistance have been expanded, investigations of Phytophthora fruit rot of pepper have been limited (23). Efforts to breed resistance to the pathogen or to develop cultural controls should include an understanding of the fruit-rot phase. The objectives of this study were to determine the relationship between Phytophthora fruit rot and fruit maturity, sugar content, and cuticle thickness. To further understand the mechanisms of resistance, experiments also were conducted to determine peroxidase activity and isozyme patterns in relation to fruit resistance and maturity. Peroxidase has been implicated in plant-disease resistance and in lignification of cell walls (8,9) and may be involved in cuticle development in peppers. Portions of this work have been previously reported (3).

MATERIALS AND METHODS

Plant material and inoculation of fruit. *P. capsici* (isolate 6012) was isolated from a pepper field near Las Cruces, NM, and maintained on corn-meal agar plates. Peppers were inoculated with *P. capsici* the same day they were harvested from the field. The procedure described by Ristaino (21) was used to promote zoospore production, except the V8-agar disks with *P. capsici* hyphae were incubated in an autoclaved sterile soil extract at

27 C. Soil for the extract was collected from a local field. Sporangia were treated with a 1-h cold shock (6 C) followed by a 1-h equilibration at room temperature. Zoospore suspensions were calibrated with a hemacytometer.

Peppers (cv. New Mexico 6-4) were planted in a plot, on 1 May 1991, south of Las Cruces, NM. Local cultural practices were used in growing and maintaining the crop. Flowers were tagged at anthesis (24 July 1991), and tagged peppers similar in age and developmental stage were harvested weekly, beginning 13 August 1991. In fruit-dip experiments, a suspension of zoospores (5×10^3 zoospores/ml of distilled deionized water) was poured into a 50-ml beaker containing one pepper fruit until 2.5 cm of the blossom end of the vertical fruit was submerged. The fruit remained in the zoospore suspension for 2 h and then was placed in a humid chamber (92% relative humidity [RH]). Disease severity was measured as the length of deteriorating tissue on the fruit 4 or 6 days later. Sixteen to 24 fruits were inoculated at each date; each fruit represented a replication.

During August and September 1992, pepper fruits at different developmental stages (immature green to red) were harvested the same day and inoculated. Twenty-five fruits were harvested from each maturity group, and 14–20 undamaged fruits from each were used in experiments. The developmental stages were distinguished according to morphological shape and color. Immature green fruits were 5- to 10-cm long and light green, characteristic of immature fruit; mature green fruits were at least 15-cm long and dark green; green/red fruits were approximately 10% red; red/green fruits were more than 50% red; and red fruits were 100% red, firm, and not dehydrated. Immature green fruits were comparable to fruits 20–33 days after flowering (DAF). Mature green fruits were comparable to fruits 41–60 DAF, and fruits turning from green to red were comparable to fruits 60–89 DAF. Inoculum drops on fruits were used during 1992 to reduce variation observed with the dip-inoculation technique (Fig. 1) used during 1991 and to more closely simulate rain splashing in the field. Zoospores (5×10^3) in a 100-μl drop of sterile distilled water were placed on 14–20 fruits from each maturity group. One-half of the fruits were wounded once, in the center with a lancet. The wound diameter was 3 mm. Wounded fruits were inoculated with the 100-μl zoospore suspension in the wounds, and sterile distilled water was used as a control. Fruits were incubated and measured as in the dip-inoculation experiments; each fruit represented a replication. Sterile water without zoospores was used as a control inoculum in all experiments.

Cuticle and pericarp measurements. Pepper fruits were harvested 26, 41, 54, 69, and 89 DAF and were stored frozen at −20 C. Fruits were thawed, and plugs approximately 0.75 cm^2 were excised, fixed in FAA for 2 h, and dried through sequential ethanol dehydrations of 50, 70, 95, and 100% at room temperature. Tissue thickness depended on the maturity stage (Fig. 2). Plugs were embedded in Spurr's low-viscosity resin (Electron Microscopy Sciences, Fort Washington, PA) and sectioned with a diamond knife on an ultramicrotome to a thickness of 1.5 μm. Sections were mounted and stained with 0.05% toluidine blue in 70% ethanol. Cuticle thickness was measured with a calibrated ocular micrometer at 400×. Four measurements were taken on each of the four sections from each pepper. Fresh (never frozen) peppers also were prepared, in an identical fashion, as controls. Cuticle thickness was not affected by freezing (data not shown).

Pericarp (flesh) thickness was measured on pepper fruits from each harvest date throughout the 1991 season. Two measurements were taken on each fruit, approximately 2.5 cm from the blossom end, and six to 12 peppers were measured on each date. Each fruit represented a replication.

Pathogen culture on fruit cell walls and water-soluble extracts. Cell-wall fragments were extracted from pepper fruits at each developmental stage (green, 10% red, 50% red, and 100% red), and water-soluble extracts were prepared from mature green and nondehydrated red fruit, as previously described (26). *P. capsici* was grown for 10 days on corn-meal agar. Three hyphal plugs (5 mm in diameter) were taken from the edge of the actively growing culture and placed in 50 ml of a minimal salt broth (Lilly-Barnett; 2 g of L-asparagine, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 0.01 mg of $FeSO_4$, 8.7 mg of $ZnSO_4$, and 3 mg of $MnSO_4$ in 1 L of water adjusted to pH 6) amended with 50 mg of fruit cell-wall fragments. Minimal salt broth without cell walls served as the control. Flasks were placed on a rotary shaker at 120 rpm for 18 days. Cultures were filtered through Whatman No. 1 filter papers under pressure in a Büchner funnel, dried at 50 C for 24 h and weighed.

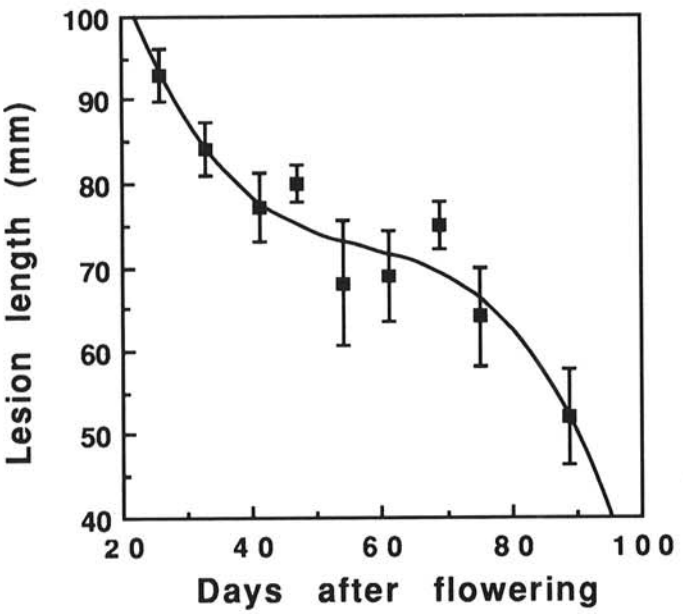

Fig. 1. Lesion length on developing peppers inoculated with *Phytophthora capsici* during 1991. Pepper fruits of the same age were dipped in a zoospore suspension (spores 5×10^3/ml) for 2 h and were removed and placed in a humid chamber (92% relative humidity) at 24 C. Sixteen to 24 peppers were inoculated on each date. The line was best described by a cubic polynomial equation: $Y = 135 - 29X + 4.8X^2 - 0.3X^3$; $R^2 = 0.24$. The line was fitted to observed values by a third-order polynomial curve of means. Vertical bars represent the standard error of the mean.

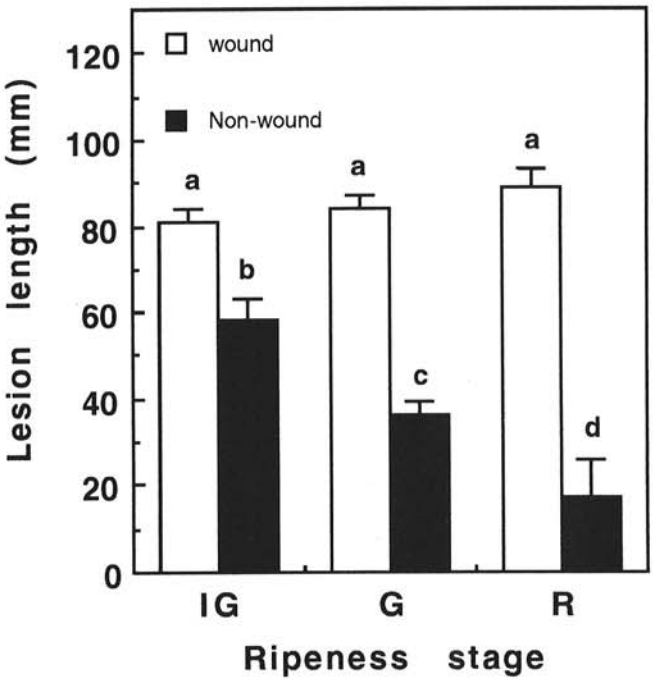

Fig. 2. Cuticle and pericarp (flesh) thickness of New Mexican-type peppers during 1991. Fruit sections measured for cuticle thickness were stained with toluidine blue and measured with a calibrated ocular micrometer at 400×. Pericarp thickness was measured on six to 12 peppers on each date. Vertical bars represent the standard error of the mean.

Cell-wall fragments were prepared according to a modified technique of Gross and Sams (6). Frozen peppers (50 g) were homogenized in 80% ethanol for 2 min, filtered through Miracloth (Chicopee Mills, Inc., New York), and rinsed with ethanol in a Büchner funnel. The residue was rinsed in 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), transferred to a 250-ml beaker, and stirred for 20 min in a phenol/acetic acid/distilled deionized water (2:1:1) solution. The suspension was refiltered through Miracloth and rinsed with 20 mM HEPES. The residue was transferred to a 250-ml beaker, suspended in a chloroform/methanol (1:1) solution, and stirred for 5 min. The suspension was filtered through Miracloth and rinsed three times with 100% acetone. The cell-wall fragments were dried for 24 h at 60 C and stored at 15 C until use.

P. capsici mycelial growth was compared on water-soluble extracts from mature green and nondehydrated red pepper fruits. Frozen pepper fruits (50 g) and sterile distilled water (500 ml) were homogenized in a blender for 3 min. The slurry was filtered sequentially through four layers of cheesecloth, Miracloth, glass filters, and Whatman No. 1 filter paper under pressure in a Büchner funnel. The extracts were transferred to 250-ml flasks and autoclaved for 20 min. Each flask, containing three plugs from an actively growing *P. capsici* culture, was incubated on a rotary shaker (120 rpm) for 10 days and weighed, as described above.

Sugar assays. Total sugars of the pericarp water-soluble extracts were measured by the phenol/H_2SO_4 method (5) and reducing sugars by the dinitrosalicylic acid method (17). Sugar and fungal-growth experiments were conducted twice, with four replications per maturity stage.

Protein extraction and electrophoresis. Proteins precipitated with acetone were extracted from pepper fruit cell walls, as previously described (4). Briefly, acetone-precipitated cell-wall powders were produced from pepper fruits at each maturity stage (green, green/red, red/green, and completely red) during the 1992 season. Frozen pepper fruits (10 g) were homogenized in 100% acetone at 24 C for 2 min. The slurry was filtered through Whatman No. 1 filter paper and washed at least three times with 100% acetone. The acetone-precipitated powders were allowed to dry at room temperature and were stored at −20 C. Samples applied to native-polyacrylamide gel electrophoresis (PAGE) were extracted with sterile deionized water (10 ml of acetone cell-wall powder to 5 ml of water). Extracts were centrifuged for 5 min at 12,000 × *g* and passed through a 0.22-μm syringe filter. A bromophenol tracking dye (5×) was added to the sample and loaded onto the gel. Protein content was measured with the BioRad protein assay (BioRad, Richmond, CA). Bovine serum albumin was used as a standard.

Protein extracts (20 μl per lane; 4 μg of total protein per lane) were applied to a 4% loading and 12% separation PAGE (0.75-mm thick) with the Laemmli (12) buffer system without sodium dodecyl sulfate, according to the instructions provided by BioRad. Gels were electrophoresed with 200 V (constant voltage) for 2 h and immediately stained for peroxidase activity with 10 mM guaiacol and 10 mM H_2O_2. A bromophenol tracking dye (5×) was added before the samples were loaded onto the gel.

Samples used to measure total peroxidase activity were extracted with 50 mM acetate buffer, pH 5, with or without 250 mM $CaCl_2$. Spectrophotometric readings (A_{470nm}) were taken 2 min after 100 μl of protein extract was added to 3 ml of 10 mM guaiacol and 10 mM H_2O_2 substrate in 50 mM acetate buffer, pH 5. Experiments consisted of three replications per maturity group.

Tissue blotting. Nitrocellulose filters (BioRad) were floated on sterile distilled water with and without 250 mM $CaCl_2$. Filters were blotted on a paper towel until semidry. Peppers were harvested according to maturity groups (immature green, mature green, and >50% red) on the same day tissue-blot experiments were conducted. Transverse sections (3 mm) of the pepper fruits were placed on the filters for 10 min. Fruit pieces were removed, and filters were stained with 10 mM guaiacol and 10 mM H_2O_2 substrate in 50 mM acetate buffer, pH 5. Similar tissue-blotting techniques were previously reported (1,24).

Data analyses. Statistical analyses were performed by the Statistical Analysis System (SAS Institute, Cary, NC). Data were subjected to regression analysis (Fig. 1) or to one-way analysis of variance. Treatment means were compared with Fisher's protected least significant difference (LSD) test or Duncan's multiple range test. All experiments were conducted three times unless otherwise indicated. Experiments were designed and analyzed as completely randomized designs; representative data sets are presented.

RESULTS

Pepper fruits harvested weekly during the 1991 season and dip-inoculated in a *P. capsici* zoospore suspension showed decreasing disease severity as the season progressed (Fig. 1). The regression line was significant at $P \leq 0.0001$ and best fit a cubic model ($P = 0.038$). Systemic lack of fit to the polynomial regression was not detected ($P = 0.53$). Fruits harvested early in the season exhibited significantly ($P \leq 0.05$) larger lesions (93 mm at 26 DAF) when compared to fruits harvested later in the season (52 mm at 89 DAF), according to a multiple-comparisons test. Fruits harvested 33 DAF were at the immature green stage of development and were completely red and beginning to dehydrate at 89 DAF.

During 1992, inoculation of fruits at different maturity stages indicated younger fruits were more susceptible to Phytophthora fruit rot than were older fruits (Fig. 3). Lesions on nonwounded fruits were significantly larger on immature green fruits than were mature green or red fruits with lesion lengths of 58, 36, and 16 mm, respectively. Wounded fruits developed larger lesions than did nonwounded fruits. There were no differences in lesion lengths among the wounded-fruit maturity groups. Disease incidence 6 days after inoculation for all wounded fruits was 100%, and disease incidence for the immature green, mature green, and red nonwounded fruits was 100, 86, and 43%, respectively.

Cuticle thickness increased as fruits aged (Fig. 2). The cuticle of immature green fruits 26 DAF was 12 μm and significantly increased to 24 μm in red fruits 50% dehydrated (89 DAF). In

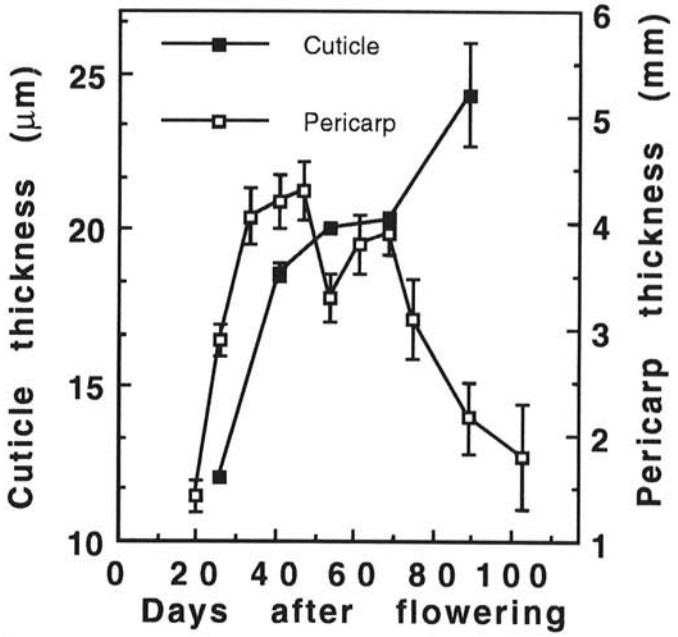

Fig. 3. Phytophthora fruit rot lesion length on wounded or nonwounded peppers at different stages of ripeness: immature green (IG), mature green (G), and red (R). Fruits were harvested and were inoculated on the same date with a 100-μl drop of 5×10^3 *Phytophthora capsici* zoospore suspension and were placed in a chamber at 92% relative humidity at 24 C. Vertical bars represent the standard error of the mean. Bars with the same letter are not significantly different according to Fisher's LSD ($P = 0.05$).

addition, as fruits matured, collapse of the epidermal cell layers was observed (data not shown). Pericarp thickness increased rapidly (0.4–4.1 mm) until 33 DAF and began to decrease 75 DAF when dehydration of the fruit began (Fig. 2). Pericarp thickness did not appear to inhibit or enhance disease severity and was similarly thick in immature green and dehydrating red fruits.

P. capsici increased in dry weight fourfold on water-soluble red pepper extracts when compared to green pepper extracts (Table 1). Growth on red fruit (89 DAF) extracts corresponded with a 2.5-fold increase in total sugar when compared to green fruit (33 DAF) extracts. A sixfold increase in reducing sugars from 33 to 89 DAF also was detected. Mycelial growth on cell-wall fragments was greater than was growth on minimal salt broth alone, but no differences were detected among fruit-maturity stages .

Peroxidase-specific activity increased as pepper fruits matured, with a twofold increase in red fruits compared to mature green fruits (Fig. 4). Treatment of the acetone powders with calcium-amended buffer enhanced peroxidase activity within each developmental group by 57, 54, and 19% in the immature green, mature green, and red groups, respectively. Average protein content at all maturity stages was 280 and 200 μg/ml for calcium-amended and nonamended acetate buffer extracts, respectively. Calcium-amended buffer appeared to remove a higher proportion of proteins from the acetone precipitants than did buffer alone.

Acidic peroxidase isozyme patterns changed as fruits matured (Fig. 5). In the immature green fruits, one peroxidase band was detected at a relative mobility (R_m) of 0.43. As fruits began to turn from green to red, two additional isozymes that moved further on the gel ($R_m = 0.50$ and 0.53) were observed at the same putative

TABLE 1. Weight of *Phytophthora capsici* hyphae grown in water-soluble pepper extracts at different fruit-maturity stages after 18 days

Fruit color[a]	Mycelial dry wt (mg)[b]	Fruit sugars (mg)[c]		pH[d]
		Total	Reducing	
Green	65 b	8.4 ±0.8	2.1 ±0.1	5.4
Red	284 a	20.1 ±1.3	13.7 ±0.6	4.6

[a] Green and red fruits were harvested 33 and 89 days after flowering, respectively.
[b] Means followed by the same letter are not significantly different according to Fisher's LSD ($P = 0.05$).
[c] Total-sugar content before fungal growth. Means are followed by standard error of the mean.
[d] pH of water-soluble extract before fungus was placed in the medium.

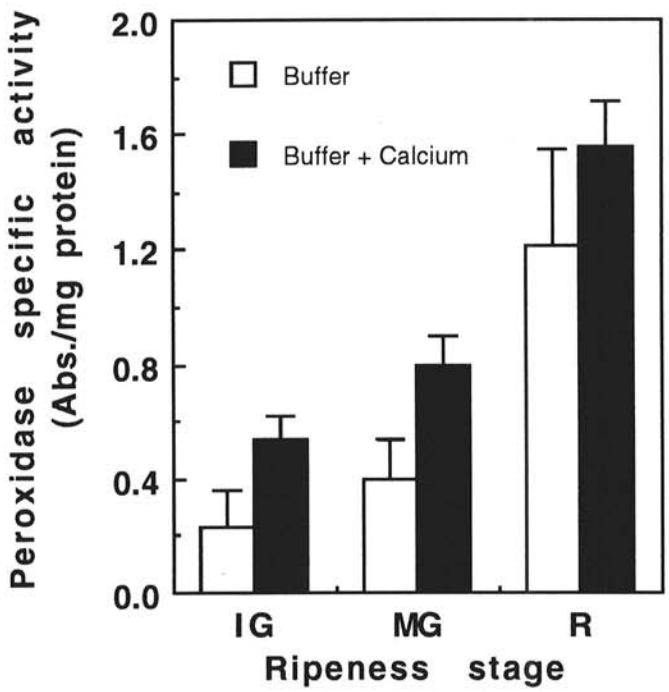

Fig. 4. Peroxidase activity of peppers at different developmental stages: immature green (IG), mature green (MG), and 100% red (R). Cell-wall acetone powders were extracted with either 0.05 M sodium acetate buffer (pH 5) or with 0.05 M sodium acetate buffer (pH 5) amended with 250 mM $CaCl_2$. Vertical bars represent the standard error of the mean.

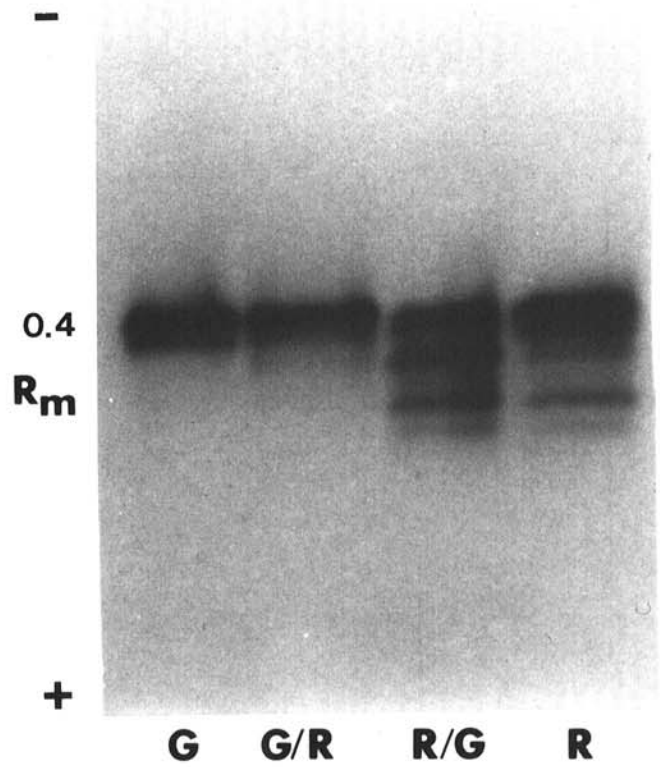

Fig. 5. Native-polyacrylamide electrophoretic gel (12%) of acidic peroxidase isozymes of peppers at different developmental stages: immature green (G), 10% red (G/R), 50–70% red (R/G), and 100% red (R). Cell-wall acetone powders were extracted with distilled deionized water. Similar results were obtained when cell walls were extracted with 0.05 M acetate buffer, pH 5, or with acetate buffer amended with 250 mM $CaCl_2$.

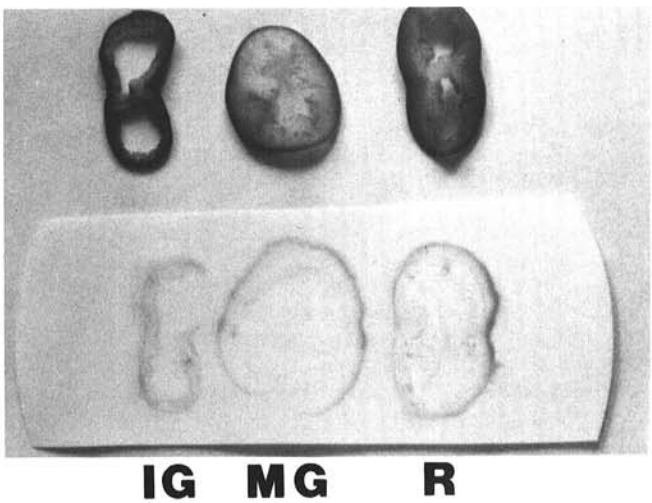

Fig. 6. Nitrocellulose tissue blotting of peppers at different developmental stages: immature green (IG), mature green (MG), and red (R). The upper row contains fruit pieces blotted on the lower row, which contains nitrocellulose filters stained for peroxidase activity. Nitrocellulose filters were pretreated with 250 mM $CaCl_2$ and were blotted on paper towels until nearly dry before fruits were applied.

locus. No other peroxidase isozymes were observed in pepper fruits. Peroxidase isozymes extracted with calcium-amended buffer exhibited the same banding patterns as those extracted with nonamended buffer or water (data not shown).

Nitrocellulose tissue-blotting experiments indicated that the peroxidase activity in immature green fruits was distributed in the pericarp and cuticular regions (Fig. 6). As fruits matured and the cuticle developed, the majority of the peroxidase activity appeared to be in the cuticular region of the mature green and red fruits. Peroxidase activity was enhanced when nitrocellulose filters were wetted in buffer amended with calcium prior to blotting fruits (data not shown).

DISCUSSION

Nonwounded pepper fruits were increasingly resistant to *P. capsici* as fruits matured and ripened. When the fruits were wounded, however, the pathogen infected and colonized green and red fruits at the same rate. *P. capsici* grown on water-soluble extracts of green or red fruits exhibited a higher level of growth on red fruit extracts than on green fruit extracts. Possible changes in the ripening fruit cell-wall fragments did not affect mycelium-culture growth in this study. Experiments conducted with *Alternaria alternata* indicated a similar growth response on pepper cell walls and water-soluble extracts (26). *A. alternata* and *P. capsici* grew best on injured red fruits that contained the highest sugar content. Therefore, the data suggest that red fruit and red fruit extracts do not inhibit growth of *P. capsici*. *A. alternata*, a wound pathogen, increased infection ability and colonization of pepper when sugars began to increase in the fruit, indicating that the fruit sugars stimulated the pathogen (26). *P. capsici*, a nonwound pathogen, did not require an increase in sugar content to infect and colonize a pepper when a wound was present.

The nonwound and wound data, plus the cuticle measurements, suggest that the physical barrier of the cuticle inhibits *P. capsici* infection, and cuticle thickness appears to be a factor in resistance to Phytophthora fruit rot. The cuticle is an important protective barrier in several host-pathogen systems (15). As bean hypocotyls age, cuticle thickness increases and is the primary factor for resistance to *Rhizoctonia solani* (25). Peach genotypes resistant to *Monilinia fructicola* exhibit a thicker cuticle, compact epidermal cells, and fewer trichomes than do susceptible peach genotypes (2).

Peroxidase also has been implicated in disease resistance in several host-pathogen systems (7–9,19,20). In our experiments, soluble-peroxidase activity increased as pepper fruits began to turn red, corresponding to the thickening of the cuticle and increased resistance of nonwounded fruit. Ionically bound peroxidase levels were similar in the maturity groups when soluble peroxidase activity was excluded. Nitrocellulose tissue blotting of cross sections of the peppers at different developmental stages showed peroxidase activity equally distributed in immature green fruits. As the fruits matured, peroxidase activity became localized in the cuticular region, suggesting peroxidase involvement in suberization and polymerization of cell wall-associated phenolic polymers of the cuticle. Calcium-amended buffer applied to acetone-precipitated powders enhanced peroxidase activity on nitrocellulose filters and in spectrophotometric analyses. Calcium apparently removed ionically bound peroxidases that were closely associated with the cell wall, as previously reported for other fruits (1,16). Acidic peroxidase isozymes also increased as fruits turned from green to red. Different isozyme patterns were not observed between calcium-extracted peroxidases and those extracted with an acetate buffer or water, further suggesting that acidic, not basic, peroxidases were bound to the cell walls. Acidic isoperoxidases may be directly involved in cell-wall lignification (7). Acidic peroxidases in pepper fruit appear to play an important role in cuticle development and therefore, disease resistance of pepper fruits.

Processing New Mexican-type peppers requires removal of the cuticle, which is a laborious and costly procedure. Breeding for a green pepper with a thin cuticle would enhance processing. A pepper with a thin cuticle, however, may result in greater levels of Phytophthora fruit rot. Cuticle thickness in relation to pepper fruit resistance should be considered when developing new pepper cultivars.

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