Changes in Methanol Evolution and Pectin Methylesterification in Resistant and Susceptible Pepper Leaves Inoculated with *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT. The hypersensitive response in resistant plants exposed to incompatible pathogens involves structural changes in the plant cell wall and plasma membrane. Cell wall changes may include pectin deesterification resulting in release of methanol. The time course of methanol production was characterized from 'Early Calwonder 20R' pepper (*Capsicum annuum* L.) leaves infiltrated with the incompatible pathogen, *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye race 1 (XCV). In the first time course experiment, leaves were infiltrated with either 10^8 colony-forming units/mL of XCV or water control. Leaf panels (1×5 cm) were excised after dissipation of water soaking, then incubated in vials at 24 °C. Headspace gas was analyzed at 6-hour intervals up to 24 hours. The rate of methanol production from resistant pepper leaves infiltrated with XCV was greatest during the first 12 hours after excision. In another experiment, leaf panels were harvested at 6-hour intervals up to 24 hours after inoculation and incubated for 12 hours at 24 °C to determine the relationship between the interval from inoculation to leaf excision and methanol production. The highest rate of methanol production was obtained when the interval between bacterial infiltration and leaf excision was 18 hours. The relationship between methanol release and changes in the degree of methylesterification (DOM) of cell wall pectin was determined in near isogenic lines of 'Early Calwonder' pepper plants resistant (20R) and susceptible (10R) to XCV race 1. Cell walls were prepared from resistant and susceptible pepper leaves infiltrated with XCV or water. XCV-treated resistant leaves had 18% DOM and $9.7 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ of headspace methanol, and the susceptible leaves had 48% DOM with $0.2 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ methanol. Susceptible and resistant control leaves infiltrated with water had 55% and 54% DOM, respectively, with no detectable methanol production. Increased methanol production in resistant pepper leaves inoculated with XCV coincided with an increase in cell wall pH. Intercellular washing fluid of resistant pepper leaves had a significantly higher pH (6.9) compared to susceptible leaves (pH 5.1) and control leaves infiltrated with water (pH 5.1). Both 10R and 20R pepper leaves infiltrated with buffer at increasing pH's of 5.1, 6.9 or 8.7 had increased methanol production. Since deesterified pectin is more susceptible to degradation, demethylation may facilitate formation of pectic oligomers with defensive signalling activity.

One problem in the pepper (*Capsicum annuum*) growing areas of the coastal U.S. is bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria* (XCV). The bacteria infect the foliage and fruits causing a reduction in fruit quality and yield (Kousik and Ritchie, 1995). Resistance to bacterial spot has been reported in certain pepper cultivars and can be used effectively to combat this disease. The sources of resistance were genetically identified as Bs1, Bs2, and Bs3, which exhibit high specificity for pathogen strains (Herbers et al., 1992; Hibberd et al., 1987a). The pathogen strains with avirulence genes *avrBs2* and *avrBs3* induced the hypersensitive response (HR) in pepper lines containing the corresponding disease resistance genes, Bs2 and Bs3 (Minsavage et al., 1990).

Plants challenged with pathogens may develop disease symptoms or exercise preformed or induced resistance. The HR is a mode of resistance involving the sudden death of plant cells attacked by a pathogenic organism (Goodman and Novacky, 1994). The HR may represent a first line of defense, sacrificing a limited number of plant cells at the infection site to directly or indirectly reduce a pathogen's potential for causing disease. Hypersensitive response symptoms were observed in the 20R line of 'Early Calwonder' resistant to XCV race 1 and disease symptoms were observed in the near isogenic 10R line, which is susceptible to XCV race 1 (Hibberd et al., 1987b). Development of the HR in resistant leaves may include structural changes in the plant cell wall and plasma membrane (Goodman and Novacky, 1994). During the HR, the changes taking place at the plant cell wall level were mainly associated with the pectic component of the primary cell wall. Pectic fragments from plant cell walls are inducers of a wide variety of defensive compounds in plant tissues at the site of pathogen attack (Ryan, 1988). Oligogalacturonides induce the production of phytoalexins in soybean [*Glycine max* (L.) Merr.] (Davis et al., 1986), parsley (*Petroselinum crispum* Mill.) (Davis and Hahlbrock, 1987), and castor bean (*Ricinus communis* L.) (Jin and West, 1984). They also play a role in the synthesis of phenolics in bean (*Phaseolus vulgaris* L.) (Tepper and Anderson, 1989) and

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proteinase inhibitors in tomato (*Lycopersicon esculentum* Mill.) (Hahn et al., 1993). Associated with the induction of defensive compounds is the stimulation of K^+ efflux and Ca^{2+} influx, followed by cytoplasm acidification and depolarization of plasma membrane potential in suspension-cultured tobacco (*Nicotiana tabacum* L.) cells (Mathieu et al., 1991).

Plants produce volatile hydrocarbons under normal growth conditions (Guenther et al., 1995). A taxonomically broad distribution of plants evolve methanol (MacDonald and Fall, 1993) with emission rates related to stomatal conductance (Nemecek-Marshall et al., 1995). Environmental stresses, including heat (Anderson, 1994) and pathogen attack (Anderson et al., 1994), can trigger elevated methanol production from leaves. Although the source of methanol is not known, heat-stressed pepper leaves that produced high levels of methanol had a lower degree of methylesterification (DOM) than controls (Anderson, 1994). Plant cell wall pectin may undergo deesterification releasing methanol in resistant pepper leaves inoculated with a pathogen. Pectin deesterification may occur due to increased pH of the cell wall solution, increased action of pectin methyltransferase (PME) (EC 3.1.1.11), or both. Alkalinization of the cell wall solution may be the result of increased leakage of electrolytes during the HR (Goodman and Novacky, 1994). Under alkaline pH conditions, there may be a simple base hydrolysis of pectin methyl esters leading to methanol release (McFeeters and Armstrong, 1984). Deesterification during the HR may also occur due to increased activity of the enzyme PME, which is present in the plant cell wall. PME activity has been reported in various plant parts such as mung bean (*Phaseolus aureus* Roxb.) hypocotyls (Bordenave and Goldberg, 1993), jelly fig (*Ficus awkeotsang* Makino) achenes (Lin et al., 1989), and *Vigna radiata* hypocotyls (Goldberg, 1984). The pH optimum for activity ranges from neutral to alkaline. PME action on esterified pectin would result in the release of methanol and render pectin chains more susceptible to the action of polygalacturonase (PG) (EC 3.1.1.15) since breakdown of the homogalacturonan region of pectin into fragments by PG is favored by a deesterified substrate (Cooper, 1983). Thus, pectin deesterification may play an important role in the signal transduction of induced disease resistance by facilitating the production of active oligomers. Therefore, hydrolysis of pectin esters, releasing methanol, may be part of an adaptive response to pathogens.

The objectives of this study were to characterize the time course of methanol production from pepper leaves infiltrated with XCV, and to determine the relationships between methanol release and changes in DOM of cell wall pectin and changes in pH of the cell wall solution in susceptible and resistant pepper leaves inoculated with XCV.

Materials and Methods

BACTERIAL CULTURE. XCV race 1 (obtained from Univ. of Florida, courtesy Dr. R.E. Stall) was stored on silica gels at -70°C according to Leben and Slesman (1982). Working cultures were periodically replenished from gels to avoid changes in growth rate and pigment production observed following extensive subculturing. Pure culture technique and preparation of inoculum as delineated by Dahlbeck and Stall (1979) was adopted with minor changes. One-week-old bacterial colonies were used to start cultures in nutrient broth. The culture flasks were maintained at 24°C for 24 h on an orbital shaker (150 rpm), then centrifuged at $5000 g_n$ for 15 min. Pellets were resuspended in sterile, distilled water and adjusted to 0.3 absorbance at 600 nm to obtain a targeted concentration of $\approx 10^8$ colony-forming units (cfu) per mL. The

actual concentration of live bacterial cells was determined by dilution plating on nutrient agar plates. A preliminary experiment was conducted to determine the dose-response relationship between bacterial inoculum concentration ($2 \pm 0.7 \times 10^5$, $2.1 \pm 0.4 \times 10^6$, $2.8 \pm 0.2 \times 10^7$, and $3.4 \pm 0.1 \times 10^8$ cfu/mL) and methanol production. The highest rate of methanol was produced with an inoculum of $3.4 \pm 0.1 \times 10^8$ cfu/mL (data not presented), which was used in all subsequent experiments. In another preliminary experiment, pepper plants were inoculated 0, 6, 12, or 18 h after the beginning of the photoperiod. The beginning of the 14 h photoperiod was designated as 0 h, hence, inoculation at 0, 6 and 12 h of the photoperiod included a light source during infiltration and subsequent incubation for 60 min in the growth chamber. Inoculating leaves 6 or 12 h into the photoperiod produced more methanol compared to 0 and 18 h (data not presented). For experimental convenience we chose to inoculate leaves 6 h into the photoperiod in all subsequent experiments.

PLANT MATERIAL, INOCULATION, AND HEADSPACE SAMPLING. Pepper seeds (obtained from Univ. of Florida, courtesy R.E. Stall) were planted in 10-L pots containing BMI planting medium (Saint-Modeste, Quebec, Canada) in a growth chamber. Plants were fertilized with Peter's (Scotts-Sierra, Marysville, Ohio) 20N-8.6P-16.6K (0.67 g-L^{-1} water) daily and maintained at 14 h light and 10 h dark periods, with 24°C day and 20°C night temperatures. Plants were watered prior to inoculation on the day of the experiment. Fully expanded leaves from 55- to 65-d-old pepper plants were syringe-injected with sterile, distilled water or a bacterial suspension (≈ 8 to $10 \mu\text{L-cm}^{-2}$) until water soaked (Klement, 1965). Depending upon the experimental objective, $1 \times 5 \text{ cm}$ leaf panels were excised at various intervals after inoculation. The incubation period from inoculation to leaf excision was always at least 60 min to allow dissipation of water soaking (Cook and Stall, 1977). One leaf panel per vial (total of three vials per treatment) was sealed in a 16.7-mL vial with a teflon-lined septum. The panels were incubated in the dark at 24°C . The incubation period for the leaf samples was based on the results of time course experiments. One milliliter of headspace gas was sampled using a gas-tight syringe and injected into a gas chromatograph (GC) (Tracor 540, Austin, Texas). The GC had a $30 \text{ m} \times 0.53 \text{ mm}$ GS-Q megabore column (J & W, Folsom, Calif.) and flame ionization detector. Helium was used as a carrier gas at the linear velocity of 93 cm-s^{-1} . The column oven was held at 60°C for 2 min, then increased 10°C/min to 140°C and held for 2 min. Temperatures of the injector and detector were 180 and 200°C , respectively. Methanol concentrations in headspace samples were determined on the basis of a standard curve derived from known concentrations of methanol (99.8%) (EM Science, Gibbstown, N.J.). The methanol peak was recognized by retention time of the methanol standard and verified by GC-mass spectroscopy (GC-MS). GC-MS was conducted according to Anderson (1994), except that the compound was identified using the selected ion monitoring procedure, choosing ions at $m/z = 31, 30, 15$, and 14 to reduce background peak interference.

TIME COURSE OF METHANOL PRODUCTION AND ELECTROLYTE LEAKAGE. To determine the time course of methanol production following bacterial inoculation, two experiments were conducted. In the first experiment, the leaf panels infiltrated with XCV at 10^8 cfu/mL or distilled water were harvested after the dissipation of water soaking (60 min after infiltration). Twelve leaf panels from each treatment were incubated at 24°C in separate vials and the headspace gas was analyzed at 6-h intervals, up to 24 h (three vials each time). In the second experiment, leaf panels were harvested at 6-h intervals up to 24 h after inoculation and the headspace gas was sampled after 12 h of incubation.

To determine the relationship between methanol release and

loss of membrane selective permeability based on leakage of electrolytes, methanol production and electrical conductivity were compared over time. Panels used to measure loss of electrolytes were treated the same as those used for headspace analysis, except they were suspended in 20 mL of distilled water in a test tube following dissipation of water soaking. Electrolyte leakage was measured using an electrical conductance meter (model 35, Yellow Spring Instrument Co., Yellow Spring, Ohio) at intervals corresponding to headspace sampling times.

CELL WALL DOM CHANGES AND METHANOL RELEASE. The relationship between cell wall DOM and methanol release was established by analyzing pepper leaf cell wall extracts and headspace gas. Cell walls were prepared as described by Huber (1991). Pepper leaves from resistant and susceptible pepper plants were infiltrated with XCV at $\approx 10^8$ cfu/mL or water. Six leaves were harvested 18 h after inoculation from both bacteria-treated and control plants. A single leaf panel (1 $\times$ 5 cm) was randomly selected from each subsample (two leaves) and incubated at 24 °C for 24 h before headspace sampling. Remaining leaf lamina (≈ 5 g for each subsample) from water- and bacteria-infiltrated leaves were diced into 0.5-cm² segments 18 h after inoculation and ground in an omnimixer with 3 volumes (v/w) of tris-buffered phenol. Samples were stirred for 1.5 h and filtered through Miracloth (Calbiochem, La Jolla, Calif.). Insoluble cell wall residues were rinsed with ≈ 2 L of 40 mM tris buffer at pH 7.0, then with deionized water to remove phenol. Residue was transferred to a sintered glass funnel and washed twice with 500 mL of 1 chloroform : 1 methanol mixture, then with acetone until white and fluffy. Cell walls were dried for 24 h at 60 °C and stored in brown bottles until analyzed.

Native sugar composition of the cell wall preparations was determined by analyzing trimethylsilyl methyl glycoside derivatives using a GC (Chaplin, 1982). Cell wall samples weighing ≈ 100 μ g were placed in a vial and 100 nmol of inositol was added as an internal standard. The sample was dried in a Speed Vac (Savant, Farmingdale, N.J.). Methanol containing 1.5 M HCl (200 μ L) and methyl acetate (50 μ L) were added to the sample prior to incubation at 80 °C overnight. About 350 μ L of tertiary butanol was added before drying under a nitrogen stream. The trimethylsilyl methyl glycoside derivative was then prepared by adding 50 μ L Trisil (Pierce Chemical Co., Rockford, Ill.) preparation (75 μ L trisil concentrate plus 225 μ L pyridine) to the sample and incubating in sealed vials at 80 °C overnight.

Just before injection into the GC, the sample was dried under a stream of nitrogen and 100 μ L isooctane (2,2,4-trimethylpentane) was added to the dried sample. An aliquot (1 μ L) was injected into a Varian 6000 GC with a 30 m DB1 fused silica capillary column (0.25 mm diameter and film thickness of 0.25 μ m, J & W, Folsom, Calif.). The flame ionization detector was set at 300 °C. The sample was injected using the cool-on-column technique with helium as the carrier gas at a linear velocity of 20 cm/s. The oven was held at 105 °C for 1 min after sample injection, then raised to 160 °C at 10 °C/min and held for 4 min, increased to 190 °C at 1 °C/min, and finally increased to 240 °C at 10 °C/min and held for 10 min. Sugar peaks were identified according to coelution with authentic standards and quantified with inositol as the internal standard.

Reduction of methyl esterified galacturonic acid residues using sodium borohydride was achieved following the procedure outlined by Maness et al. (1990). Samples were then methanolized and trimethylsilylated, followed by GC analysis as described above. Degree of methyl esterification was calculated as the ratio of galactose produced by reduction to the total galacturonic acid as follows:

$$\text{DOM} = [\text{GALACTOSE (R)} - \text{GALACTOSE (N)}] \times 100 / [\text{GALACTOSE (R)} - \text{GALACTOSE (N)} + \text{GALACTURONIC ACID (R)}]$$

where (R) represents the sample reduced by sodium borohydride and (N) represents the native sample without sodium borohydride. Therefore, [galactose (R) – galactose (N)] represents methyl esterified galacturonic acid in the native samples (Maness et al., 1990).

THE pH OF INTERCELLULAR WASHING FLUID AND METHANOL PRODUCTION. Bacterial suspensions and water controls were syringe-infiltrated into resistant and susceptible pepper leaves. A single leaf panel was obtained from each leaf 18 h after infiltration for head space analysis and was incubated at 24 °C for 12 h. The rest of the leaf lamina was infiltrated with distilled water to rinse intercellular spaces (Klement, 1965). Leaf samples were then centrifuged at 30 g_n for 10 min and the pH of recovered intercellular washing fluid (IWF) was measured using a small pH probe (model PHR-146, Lazar Research Laboratories Inc., Los Angeles, Calif.).

BUFFER INFILTRATION. Resistant and susceptible pepper leaves were syringe-infiltrated with 100 mM 2-[N-morpholino] ethanesulfonic acid (MES) buffer at pH 5.1 or 6.9, 100 mM bis-tris propane (BTP) at pH 8.7, or water control. The pHs of 5.1 and 6.9 were selected to correspond with previously measured IWF pH of bacteria-infiltrated susceptible and resistant pepper leaves, respectively. A leaf panel (1 $\times$ 5 cm) was excised from each of three leaves per treatment combination immediately following infiltration and incubated in sealed vials for 12 h at 24 °C in the dark before headspace analysis by GC. Data were expressed on an area basis, rather than fresh mass, due to the added buffer.

EXPERIMENTAL DESIGN. The experimental data were analyzed as a randomized complete block design. The plants were considered blocks in all experiments. Subsamples ($n = 3$) consisted of 5 g of leaf tissue in DOM experiments, and leaf panels in other experiments. The experiments were replicated three times on different dates. Data were analyzed by analysis of variance using the general linear model (SAS Institute, Cary, N.C.). If the interaction of bacterial treatment by time of incubation or time of harvest was significant, then the main effects of bacterial treatment and the interaction were combined and trends were calculated for each bacterial treatment (Snedecor and Cochran, 1971). Buffer infiltration data were further analyzed using orthogonal contrasts.

Results and Discussion

TIME COURSE OF METHANOL PRODUCTION AND ELECTROLYTE LEAKAGE. There was a significant interaction ($P < 0.01$) in methanol production between inoculation of leaves with bacteria or water and the duration of incubation (Table 1). Analysis of variance by treatment revealed that methanol production from water-infiltrated leaves did not change with time, but when leaves were infiltrated with bacteria, methanol production increased curvilinearly with time (Table 1). The rate of increase was the greatest between 6 h and 12 h of incubation (Fig. 1).

Electrolyte leakage of pepper leaves showed a significant interaction ($P < 0.01$) between water- and bacteria-infiltrated leaves with time (Table 1). Bacteria-inoculated leaves and water-treated leaves showed significant ($P < 0.01$ and 0.05, respectively) linear increases in leakage between 0 and 24 h. However, the slope for the bacteria-inoculated leaves was greater than the water-infiltrated leaves (Fig. 1). Although similar results were reported (Cook and Guevara, 1984), the time necessary for collapse of host cells varies for each combination of resistant pepper line and incompatible race of bacteria (Hibberd et al., 1987b). The basis for

Table 1. General linear model analysis of the effect of incubation time on methanol production and electrolyte leakage in pepper leaves infiltrated with *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye race 1 (XCV) or water.

Source	df	Sum of squares for methanol	Sum of squares for electrolyte leakage
Treatment ²	1	11814.1**	13542.4**
Time	4	60782.2**	17778.5**
Replication	2	632.3 ^{NS}	1294.9*
Subsamples	2	8798.5*	1037.4 ^{NS}
Treatment × time	4	71066.1**	7643.5**
Bacteria linear	1	136333.5**	23934.2**
Bacteria quadratic	1	6019.6**	236.7 ^{NS}
Bacteria cubic	1	73.4 ^{NS}	48.7 ^{NS}
Control linear	1	39.4 ^{NS}	976.4*
Control quadratic	1	0.4 ^{NS}	181.2 ^{NS}
Control cubic	1	29.6 ^{NS}	21.4 ^{NS}
Error	18	614.1	166.3

²With or without bacteria.

^{NS}, *, ** Nonsignificant or significant at $P \leq 0.05$ or 0.01 , respectively.

Fig. 1. Effect of the time of incubation on methanol production and electrolyte leakage from pepper leaves inoculated with *Xanthomonas campestris* pv. *vesicatoria*. Each point is the mean of nine observations and vertical bars are the standard error of the mean.

electrolyte leakage after infiltration of HR-inducing bacteria into pepper leaves appears to be extensive damage to the host plasma membrane (Goodman and Novacky, 1994).

The duration between inoculating leaves with HR-inducing bacteria and harvesting leaves for headspace analysis affected methanol production (Table 2). Methanol release in bacteria-infiltrated leaves increased linearly, but infiltration with water did not affect methanol production (Table 2, Fig. 2). Leakage of electrolytes from bacteria-treated leaves also differed significantly ($P < 0.01$) from water-infiltrated leaves (Table 2). Electrolyte leakage was curvilinearly related to harvest time in bacteria-treated leaves, and was not affected by water-treated pepper leaves (Table 2). The highest rate of leakage was obtained when leaves were harvested 18 h and 24 h after bacterial inoculation (Fig. 2).

DOM CHANGES AND METHANOL RELEASE. There was a significant ($P < 0.05$) interaction between pepper genotype and bacteria treatments. The DOM of cell wall pectin was lower (18%) in resistant plants infused with XCV when compared to susceptible

plants infused with XCV (48%) (Fig. 3). Methanol production ($9.7 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) was higher in the resistant plant–XCV combination than the susceptible combination ($0.2 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). Deesterification of pectin from cell walls of pepper leaves was enhanced in the resistant plant infiltrated with XCV. In water-infiltrated leaves, DOM of resistant and susceptible leaf cell wall pectin was 54% and 55%, respectively, and methanol production was undetectable.

Pectin deesterification may be influenced by the activity of the cell wall enzyme PME (Tieman et al., 1992) and/or base hydrolysis of pectin methyl esters (McFeeters and Armstrong, 1984). Deesterification in resistant pepper plants may have been due to augmented PME activity and/or increased pH of the cell wall solution. Alkalinization of the cell wall solution is associated with HR symptom development (Atkinson and Baker, 1989). An important role of deesterification in HR development may be facilitating formation of pectin oligomers since deesterified pectin is a substrate for PG. Polygalacturonase action on the pectin chain accumulates short fragments which have the capacity to elicit the HR (Ryan, 1988). In resistant pepper plants, enhanced PG activity

Table 2. General linear model analysis of the effect of harvest time on methanol production and electrolyte leakage in pepper leaves infiltrated with *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye race 1 (XCV) or water.

Source	df	Sum of squares for methanol	Sum of squares for electrolyte leakage
Treatment ²	1	2401.0**	99385.1**
Time	4	555.0 ^{NS}	28795.7*
Replication	2	469.7 ^{NS}	2659.7 ^{NS}
Subsamples	2	22.3 ^{NS}	365.5 ^{NS}
Treatment × time	4	568.8**	29461.4**
Bacteria linear	1	810.9**	46369.5**
Bacteria quadratic	1	101.6 ^{NS}	156.6 ^{NS}
Bacteria cubic	1	99.3 ^{NS}	7762.6*
Control linear	1	0.5 ^{NS}	51.4 ^{NS}
Control quadratic	1	0.02 ^{NS}	84.6 ^{NS}
Control cubic	1	0.06 ^{NS}	30.0 ^{NS}
Error	18	98.4	1982.0

²With or without bacteria.

^{NS}, *, ** Nonsignificant or significant at $P \leq 0.05$ or 0.01 , respectively.

Fig. 2. Effect of time of harvest on methanol production and electrolyte leakage from pepper leaves inoculated with *Xanthomonas campestris* pv. *vesicatoria*. Each point is the mean of nine observations and vertical bars are the standard error of the mean.

Fig. 3. Cell wall pectin degree of methyl esterification and headspace methanol produced by resistant and susceptible pepper leaves treated with *Xanthomonas campestris* pv. *vesicatoria* (bacteria) or water (control). Leaves were harvested 18 h after infiltration and incubated for 24 h before headspace analysis. Values are means of nine measurements.

Fig. 4. Intercellular washing fluid pH and methanol production by resistant and susceptible pepper leaves infiltrated with *Xanthomonas campestris* pv. *vesicatoria* (bacteria) or water (control). Leaves were harvested 18 h after infiltration and incubated for 12 h before headspace analysis. Intercellular washing fluid was prepared immediately after harvest. Values are means of nine measurements.

may result in the production of pectin oligomers which can elicit the HR. In the case of susceptible pepper plants, slow deesterification presumably slows an increase in PG substrate availability, which apparently progresses more rapidly during an incompatible interaction. Pectate lyase produced by XCV has pectolytic activity on pepper, but does not have an essential role in pathogenicity (Beaulieu et al., 1991). However, we can not rule out the possibility of a role of pectate lyase in the synthesis of elicitor-active oligomer fragments in resistant pepper leaves during the development of the HR. Since pectate lyase also requires a deesterified substrate, it may act similarly to PG on resistant and susceptible isolines.

THE pH OF INTERCELLULAR WASHING FLUID AND METHANOL PRODUCTION. There were significant interactions between pepper geno-

type and bacteria treatment for IWF pH ($P < 0.01$) and for methanol emission ($P < 0.05$) (Fig. 4). The pH of the resistant plant-bacteria combination (6.9) was higher than the susceptible plant-bacteria combination (5.1). Methanol release in the resistant plant and bacteria combination ($15.8 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) was higher than the susceptible plant and bacteria combination ($0.3 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). In the absence of bacteria, the IWF pH of resistant plants was 5.2, and the methanol release was $0.1 \text{ nmol} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$.

Introducing XCV to resistant pepper leaves increased the IWF pH and stimulated methanol release. Alkalinization of the IWF pH was probably caused by an increase in the leakage of electrolytes such as K^+ and entry of H^+ into the cytoplasm (Atkinson and Baker, 1987). Potassium efflux and H^+ influx are early events in the HR response. Intercellular washing fluids of pepper leaves infiltrated with XCV collected over a period of 96 h after HR initiation exhibited a 10-fold increase in K^+ content (Sinclair et al., 1970).

BUFFER INFILTRATION. Methanol release in both pepper genotypes (10R and 20R) increased with increasing pH of the buffer solutions from 5.1 to 8.7 (Fig. 5). Anderson (1994) reported similar results using 'Early Calwonder' pepper leaf disks infiltrated with buffer solutions. There were significant orthogonal contrasts ($P < 0.01$) between pH 5.1 vs. 6.9 as well as between pHs 5.1 and 6.9 vs. 8.7. There was no significant interaction between plant type and buffer pH. Elevated methanol release from pepper leaves at a higher pH is consistent with our previous results where methanol production was greater when pH of the IWF was 6.9, compared with 5.1 (Fig. 4). Different experimental procedures resulted in different units for expressing data from buffer and bacteria experiments. If data from buffer experiments are converted to the same units as bacteria experiments using a representative mass to area ratio for pepper leaves, three to four times as much methanol was measured in buffer experiments at pH 6.9, compared with the bacteria treatment that resulted in an IWF pH of 6.9. The apparently greater magnitude of methanol release from buffer infiltration compared with bacterial treatment may be due to a greater number of affected cells in buffer treatments or differences in effective treatment time. Treatments that increase pH to 6.9 release only a fraction of the available methanol based on values from the buffer treatment at pH 8.7.

Fig. 5. Effect of pH of infiltrating solution on methanol release in pepper genotypes. Values are means of nine measurements. Vertical bars are the standard error of the mean.

Methanol emission was also observed from isolated cell walls of cucumber (*Cucumis sativus* L.) and sugarbeet (*Beta vulgaris* L.) treated with NaOH, which hydrolyzed the methyl esters of cell wall pectin (McFeeters and Armstrong, 1984). NaOH added to the cell wall samples raised the pH to alkaline conditions, favoring hydrolysis of methyl ester groups. Accelerated methanol production from XCV-inoculated resistant pepper leaves at pH 6.9 may be the result of pectin deesterification due to a simple base hydrolysis. It may also be due to increased activity of PME at a pH closer to its optimum of ≈ 7.5 (Nari et al., 1991).

Initial attempts at measuring PME activity by infiltrating ANTS (8-aminonaphthalene-1,3,6-trisulfonate)-labelled pectin oligomers into pepper leaves and then analyzing the recovered labelled pectin by electrophoresis, a modification of the PG assay described by Zhang et al. (1996), revealed that pepper leaves had extensive PME activity (unpublished data). Although, we did not detect any difference in PME activity between susceptible and resistant pepper leaves infiltrated with XCV, additional studies will be required to resolve this question.

In summary, infiltration with concentrations of 10^6 cfu/mL or higher of XCV produced more methanol from excised leaf panels than water-infiltrated controls. In a time course experiment, the rate of methanol production in resistant pepper leaves infiltrated with XCV was greatest during the first 12 h after excision. Electrical conductivity increased linearly in both bacteria and control treatments, but the slope was significantly higher in leaf disks infiltrated with bacteria. The highest rate of methanol production was obtained when the interval between bacterial infiltration and leaf excision was 18 h. Changes in electrical conductivity over time paralleled changes in methanol evolution. Greater pectin deesterification and methanol evolution were observed in resistant pepper leaves inoculated with XCV, compared to susceptible leaves. Intercellular washing fluid from resistant leaves had a higher pH than IWF from susceptible leaves following bacterial inoculation. Methanol evolution increased from both resistant and susceptible leaves infiltrated with buffer solutions at successively higher pH. Increased pectin deesterification and concomitant methanol release in the resistant pepper-XCV combination may be related to increased pH in the cell wall environment via changes in activity of enzymes such as PME. Additional studies will be required to determine whether differential deesterification of pectin in resistant and susceptible isolines is a general phenomenon and whether it is related to induction of plant defenses.

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