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Ultrastructure of Tomato Leaf Tissue Treated with the Pseudomonad Phytotoxin Coronatine and Comparison with Methyl Jasmonate

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Coronatine is a chlorosis-inducing phytotoxin produced by several pathovars of *Pseudomonas syringae*. Light and electron microscopy were used to determine the effect of coronatine on tomato leaf tissue. Although the structural integrity of cells in coronatine-treated chlorotic leaves was maintained, the toxin induced thickening of cell walls and shrinkage of chloroplasts. Coronatine also stimulated the accumulation of large cubical protein particles and smaller proteinaceous spheres in leaf parenchyma cells; this was coincident with a dramatic increase in proteinase inhibitor activity. Growth of *P. syringae* pv. *tomato* was more pronounced on coronatine-treated tissue than on healthy tissue, despite the accumulation of proteinase inhibitors in toxin-treated tissue. Tomato leaf tissue infected with a coronatine-producing strain of *P. s.* pv. *tomato* exhibited symptoms similar to tissue treated with the purified toxin, while tissue infected with a COR⁻ mutant showed symptoms similar to untreated healthy tissue. Methyl jasmonate and coronafacic acid, compounds structurally similar to coronatine, induced the accumulation of proteinase inhibitors but did not affect chloroplast size or induce chlorosis. Our results show that the virulence factor coronatine does not cause massive cellular destruction in the tomato leaves. Furthermore, the proteinase inhibitors induced in the plant do not slow pathogen growth, and coronatine does not act solely by mimicking methyl jasmonate.

Additional keywords: chymopapain, chymotrypsin, jasmonic acid, plant defense.

Coronatine is a non-host-specific phytotoxin produced by several members of the *Pseudomonas syringae* group of pathovars. The toxin acts as a virulence factor in *P. syringae* pv. *tomato*, allowing the organism to multiply to a higher population density and develop larger lesions than mutant strains unable to produce the toxin (Bender et al. 1987). The most prominent symptom observed in leaf tissue treated with coronatine is an intense spreading chlorosis; this has been attributed to a loss of chlorophylls a and b in tobacco (Kenyon and Turner 1990). Several recent reports indicate that coronatine functions as a molecular mimic of octadecanoid plant signaling molecules. For example, both coronatine (Fig. 1A) and methyl jasmonate (Fig. 1C) induced the accumulation of a proteinase inhibitor in tomato leaves and inhibited root growth in *Arabidopsis* seedlings (Feys et al. 1994). Similarly, *Arabidopsis* mutants insensitive to coronatine's effects were also insensitive to the effects of methyl jasmonate (Feys et al. 1994). Furthermore, coronatine was highly active in several jasmonate-selective bioassays, with activity and structure most closely related to 12-oxo-phytodienoic acid, a precursor of jasmonate (Weiler et al. 1994).

At the ultrastructural level, most chlorosis-inducing phytotoxins typically disrupt plastid membrane morphology or integrity. The pseudomonad toxins tagetitoxin and tabtoxinine-B-lactam prevent chloroplast development and disrupt existing chloroplast membranes (Lukens and Durbin 1985; Jutte and Durbin 1979; reviewed in Durbin 1991). The phytotoxin cercosporin disrupts the integrity of chloroplasts, mitochondria, and the plasmalemma (Steinkamp et al. 1981),

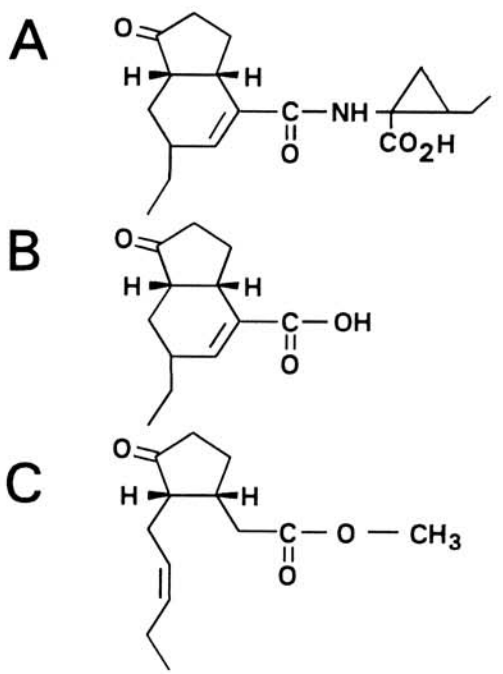

Fig. 1. Structures of **A,** coronatine; **B,** coronafacic acid, and **C,** methyl jasmonate.

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whereas tentoxin causes multiple effects on chloroplast and plasmalemma membrane activity (reviewed in Klotz 1988). The host-specific toxins victorin and helminthosporoside disrupt chloroplast membranes and the plasmalemma (Luke et al. 1966; Strobel et al. 1972), while the effects of the volatile plant growth hormone ethylene, which also induces chlorosis, are related to a destruction of chloroplast membranes (Fukada and Toyama 1982; Ronning et al. 1991). Not all phytotoxins cause chlorosis by disrupting membranes; phaseolotoxin inhibits chlorophyll biosynthesis by inducing an arginine deficiency (Turner and Mitchell 1985), whereas rhizobitoxine induces a methionine deficiency (Durbin 1991).

Coronatine's effects on membrane integrity and cell ultrastructure have not been previously investigated. The present study describes changes in tomato leaves associated with the application of coronatine, methyl jasmonate, and coronatine-producing and nonproducing-producing strains of *P. syringae* pv. *tomato*.

RESULTS

Healthy tissue.

In H_2O-treated healthy tissue observed 8 days after inoculation, primary leaves were dark green, with normal structure and ultrastructure (Figs. 2A,C and 3C). Epidermal and mesophyll cells contained large central vacuoles which appeared empty. Chloroplasts, mitochondria, vacuoles, and granular cytoplasm were visible and intact in all mesophyll cells. The application of H_2O by rolling a wet cotton swab over the leaflet or by applying H_2O directly to the leaf surface led to no observable differences in leaf structure or ultrastructure.

Coronatine-treated tissue.

In contrast to H_2O-treated tissue, coronatine-treated tissue showed a diffuse chlorosis extending approximately 5 mm from the inoculation site when observed 8 days after inoculation. The appearance of the coronatine-treated tissue was accompanied by a concomitant loss of chlorophylls a and b (Table 1). Leaf thickness, cell number, and cell dimensions were similar for both healthy and coronatine-treated, chlorotic tissue (Fig. 2A and B); however, the epidermal cell walls were consistently thicker in coronatine-treated leaves (compare Figs. 2C,D and 3A,B). Although chloroplasts in coronatine-treated tissue were significantly smaller, often had reduced starch deposits, and stained more intensely than those in healthy tissue (compare Figs. 2C,D and 3C,D), the membrane systems appeared to be intact (Fig. 3E,F). Mitochondria were similar in both tissues.

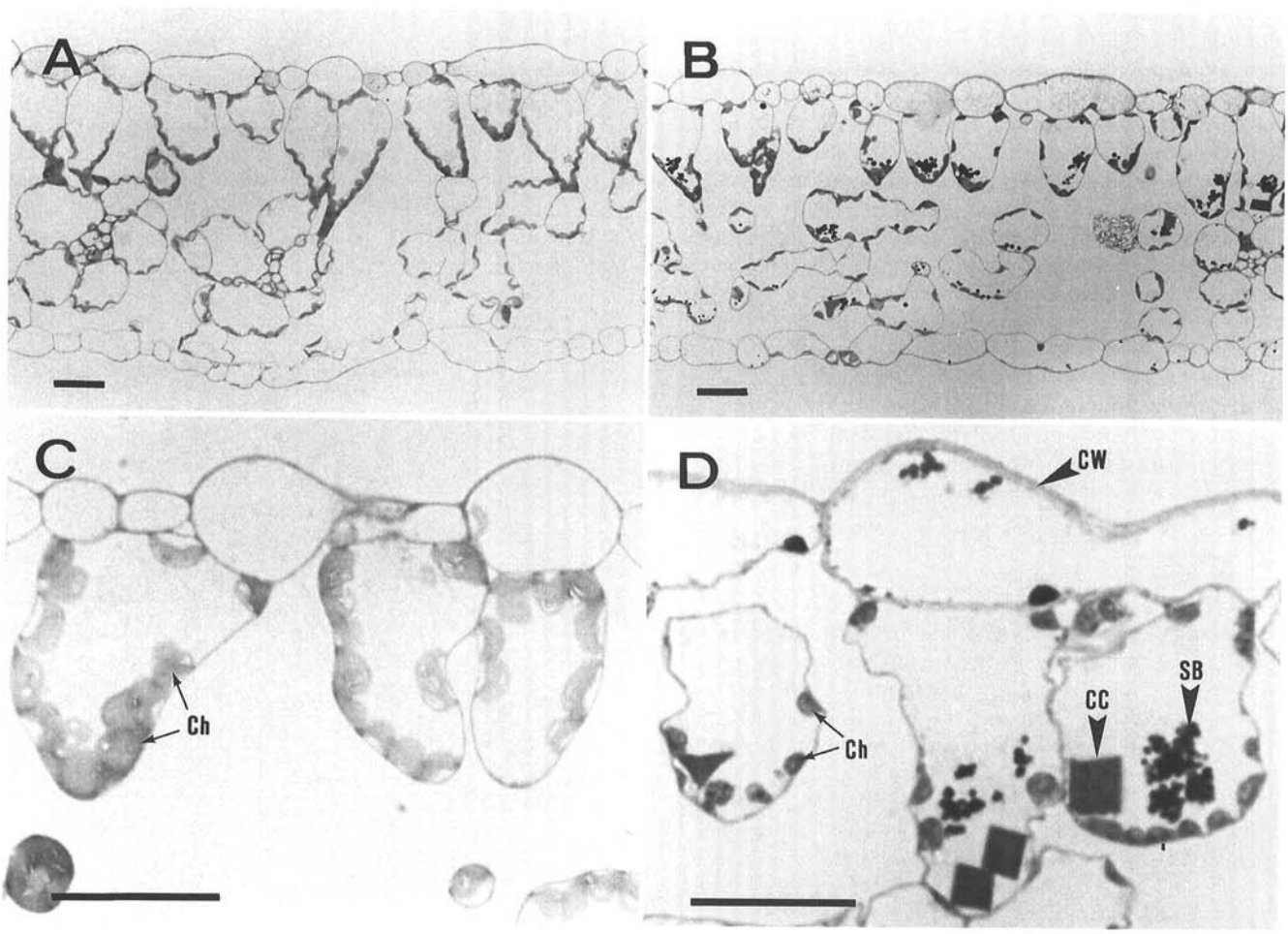

Fig. 2. Effects of coronatine on tomato leaf tissue 8 days after inoculation. **A, C,** Tissue treated with sterile water. **B, D,** Tissue treated with 125 ng of coronatine showing shrunken chloroplasts (Ch), spherical protein bodies (SB), cubical protein crystals (CC), and thickened cell wall (CW). Coronatine-treated tissue (**B, D**) absorbs stain much more strongly than healthy tissue (**A, C**). Bars = 25 μm.

Coronatine-treated tissue contained cubical and spherical particles in the mesophyll cells which were visible at both the light and EM level. The cubical particles (Figs. 2D, 4A, and 5) were large (often up to 6 μm per side), moderately electron-dense, granular, and were apparently surrounded by numerous ribosomes and a thin layer of cytoplasmic ground substance (Fig. 4A). The spherical particles (Figs. 2D, 4B, and 5) were visible as electron-dense granular spheres and were present in the vacuoles of mesophyll cells treated with coronatine. These spherical particles were clustered into aggregates, with each sphere up to 1 μm in diameter. Both particle types were flexible in thin section and stained

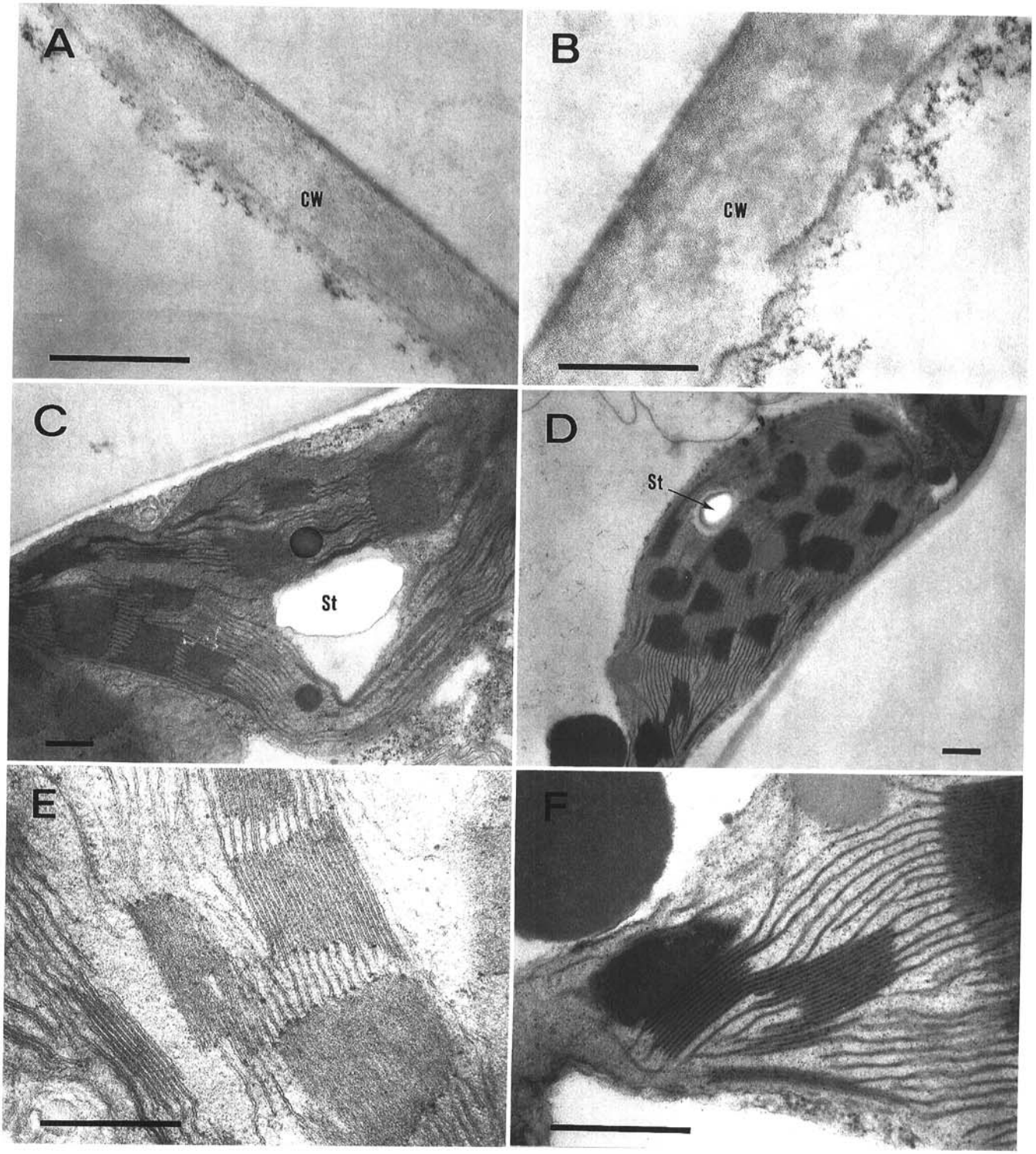

Fig. 3. Comparison of tissue treated with sterile water (**A, C, E**) and 125 ng of coronatine (**B, D, F**). **A, B,** coronatine induces thickening of the upper epidermal cell walls (CW). **C, D,** Typical chloroplasts showing starch deposits (St) and intact membrane systems. **E, F,** Detail of chloroplast membrane systems showing enhanced staining in coronatine-treated tissue. Bars = 0.5 μm.

intensely with Coomassie brilliant blue R-250, indicating a proteinaceous composition. Assays for proteinase inhibitors indicated a substantial accumulation of chymopapain and chymotrypsin inhibitors in coronatine-treated tissue (Table 1).

To determine when ultrastructural changes first occurred, tissue was fixed at 0, 2, 5, 8 and 12 days after coronatine was applied (Fig. 6). Chlorosis was first visible 4 days after inoculation, and the treated sites were fully chlorotic at 6 days. There was a significant increase in stain uptake 2 days after inoculation (Fig. 6). Spherical and cubical protein bodies were first visible 2 days after inoculation and increased in both size and number at 5 and 8 days. No increase in the number of protein bodies was observed between 8 and 12 days. Between 2 and 5 days, the chloroplasts began to shrink and settle to the lower portion of the palisade mesophyll cells; there were no further changes in chloroplast appearance after 8 or 12 days. Thickening of epidermal cell walls was especially pronounced at 8 and 12 days. Starch deposits were visible in chloroplasts at all sampling points.

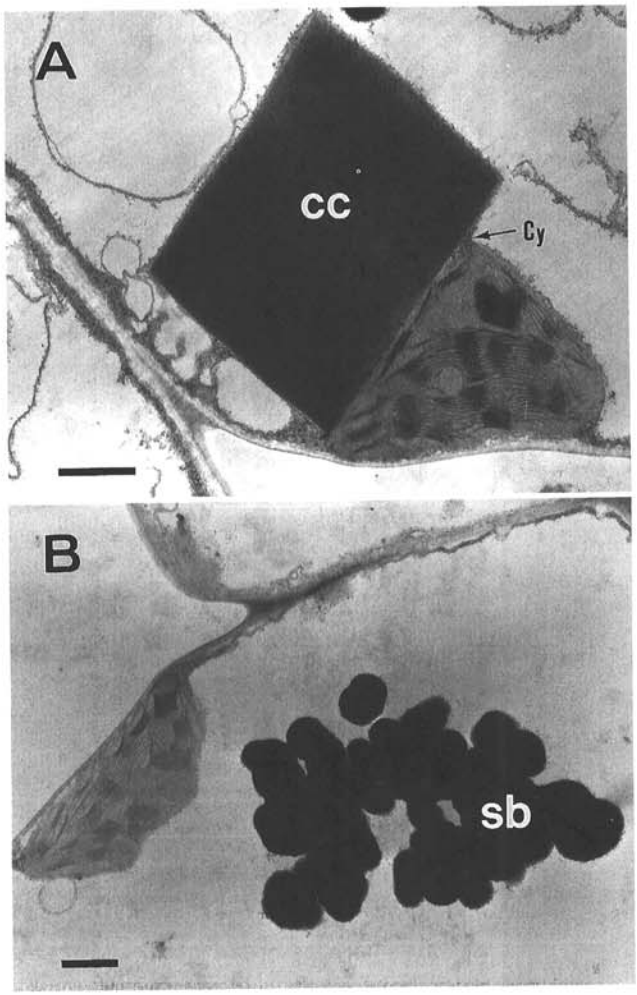

Fig. 4. Ultrastructural details of, **A,** a cubical proteinaceous crystal (cc), and, **B,** a spherical protein body cluster (sb), found in leaf tissue treated with 125 ng of coronatine. Note cytoplasm (Cy) surrounding crystal. Identical structures were observed in tissue treated with 12,500 ng of methyl jasmonate. Bars = 1 µm.

Systemic effects of coronatine treatment were monitored by sampling several sites on a plant on which a single leaflet was treated with the toxin (Table 2). Although cubical crystals and chlorosis were observed only in the treated leaf, spherical protein bodies were apparent in all leaves sampled.

Lesions induced by PT23.21, a Cor⁻ strain.

P. s. pv. *tomato* PT23.21, a coronatine nonproducer, induced necrotic lesions substantially smaller than those observed with PT23.2 (compare Fig. 7A and B). At 8 days, these lesions were approximately 0.2 to 0.5 mm in diameter and were not surrounded by a chlorotic area. Bacterial populations were restricted to the area within the necrotic lesion and to the external surface of the lower leaf cuticle (Fig. 7A). Parenchyma and epidermal cells immediately adjacent to the lesion were identical to healthy tissue when observed by light (Fig. 7A) and electron microscopy.

Lesions induced by PT23.2, a Cor⁺ strain.

At 8 days after inoculation, lesions induced by *P. s.* pv. *tomato* PT23.2 had a sharply defined necrotic center with a diameter of approximately 1 mm. Surrounding these lesions were chlorotic areas with diffuse margins extending approximately 2 mm from the lesion center. Colonization of tomato leaves by PT23.2 (Fig. 7B) was similar to PT23.21 (Fig. 7A) because bacterial cells were restricted to the necrotic lesion and to the external surface of the lower leaf cuticle. Although bacterial cells were almost always absent from the chlorotic tissue surrounding the lesion (Fig. 7B), in one section some bacterial cells were observed in the intercellular spaces surrounding a leaf vein 2.5 mm from the

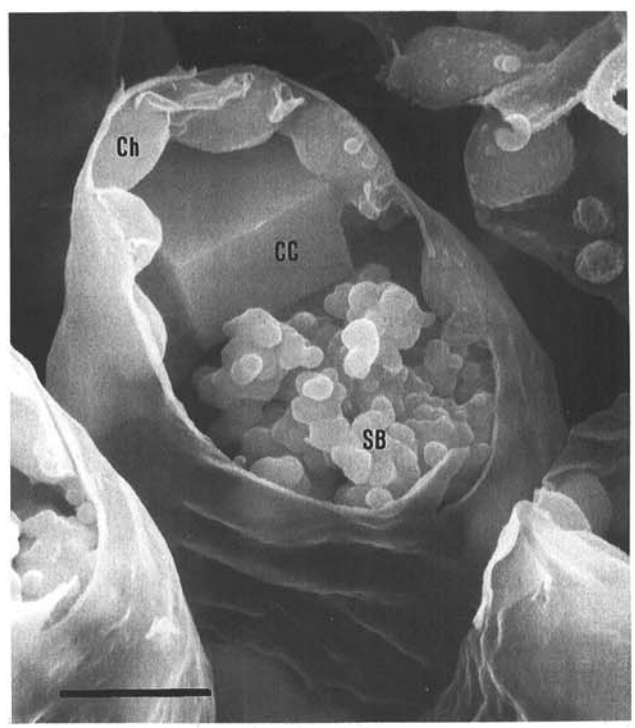

Fig. 5. Scanning electron micrograph of a palisade mesophyll cell as viewed from below, with a portion of the cell wall removed. Note chloroplasts in close contact with cell wall (Ch), spherical protein bodies (SB), and a large cubical crystal (CC). Bar = 5 µm.

lesion center (data not shown). The chlorotic tissue surrounding the necrotic lesion exhibited the same symptoms observed in coronatine-treated tissue when viewed by both light (Fig. 7B) and electron microscopy (data not shown). The chlorotic tissue surrounding the necrotic lesions contained shrunken chloroplasts, thickened walls, and a massive accumulation of spherical protein bodies and cubical crystals.

Leaves inoculated with methyl jasmonate.

To determine the optimal amount of methyl jasmonate (MeJas) necessary to induce symptoms similar to coronatine, a 10-fold dilution series was applied to different leaflets of a single leaf. Coronatine (125 ng) was applied to one leaflet, and 125, 1250, 12,500, and 125,000 ng of methyl jasmonate to the remaining leaflets. The highest level of MeJas (125,000 ng) caused an intense water-soaking after 2 h; within 18 h, the entire leaflet was water-soaked, wrinkled, and wilted. The damaged leaflet was removed at this time. All other levels of jasmonate induced no visible symptoms even at 10 days after inoculation, when the coronatine-treated leaflet had long since become chlorotic.

Leaves treated with 12,500 ng MeJas (the highest non-phytotoxic level) were sampled and fixed 8 days after inoculation. These leaves were not chlorotic, and chlorophyll content was not significantly different from water-treated leaf tissue (Table 1). Light micrographs (Fig. 8A) indicated that the MeJas-treated tissue contained both the cubical crystals and spherical protein bodies but was otherwise similar to the water-treated control tissue. Assays for proteinase inhibitors indicated a substantial accumulation of chymopapain and chymotrypsin inhibitors in response to methyl jasmonate (Table 1). Unlike coronatine-treated tissue, tomato leaves treated with MeJas did not exhibit differences in chloroplast size or staining, and the thickness of cell walls was similar to healthy tissue.

Cellular structure of coronafacic acid-treated leaves, wounded leaves, and naturally senescent tissue.

Chlorosis was not observed and chlorophyll content was not reduced (Table 1) in leaves treated with 75 ng of coronafacic acid (CFA), a precursor of coronatine and structural analogue of methyl jasmonate (Fig. 1). Light microscopy (Fig. 8B) revealed that CFA-treated tissue was similar in appearance to untreated tissue, except for the slight accumulation of spherical protein bodies. Assays for proteinase inhibitors indicated a substantial accumulation of chymopapain and chymotrypsin inhibitors (Table 1).

Table 1. Chlorophyll content and chymopapain and chymotrypsin inhibitor activity in tomato tissue treated with coronatine, coronafacic acid, and methyl jasmonate

Treatment[a]	Chlorophyll a (% of healthy)[b]	Chlorophyll b (% of healthy)[b]	Chymopapain inhibitor activity[c] (mg inhibited ml^{-1})	Chymotrypsin inhibitor activity[c] (μg inhibited ml^{-1})
Coronatine	54.2 ± 30.4	51.2 ± 32.3	333.3 ± 144	208.3 ± 72
Coronafacic acid	103.1 ± 34.5	103.4 ± 35.9	145.8 ± 95	83.3 ± 36
Methyl jasmonate	102.4 ± 30.6	106.3 ± 24.9	208.3 ± 72	104.2 ± 36
Sterile H_2O	100.0	100.0	20.8 ± 36	ND

[a] Tomato plants were treated with 125 ng of coronatine, 75 ng of coronafacic acid, 12,500 ng of methyl jasmonate, or sterile water. After 8 days, the treated leaves were analyzed for chymopapain or chymotrypsin inhibitor activity using the method of Akers and Hoff (1980), and the chlorophyll content of the crushed leaves was measured.

[b] Chlorophylls a and b were measured by monitoring absorbance at A_{665} and A_{649} nm, respectively, of leaf tissue extracted in acetone:ethyl acetate (1:1, v/v). Values represent the percentage of chlorophyll present relative to healthy tissue and represent the means and standard error values for four replicates.

[c] Values indicate enzyme inhibitor activity and represent the maximum concentration of enzyme which was fully inhibited by extracts from the treated leaf. Means and standard errors are based on three replicates. ND = inhibitor activity not detected.

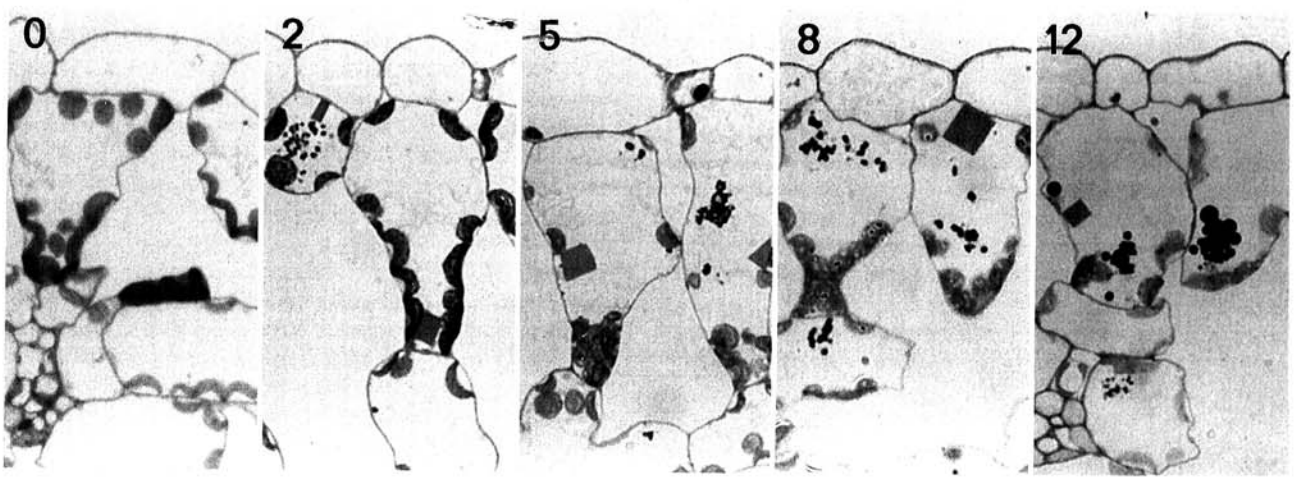

Fig. 6. Time course of symptoms induced by coronatine. Leaf tissue was fixed and processed at 0, 2, 5, 8, and 12 days after inoculation with 125 ng of coronatine and subsequently viewed by light microscopy. Representative views from each sampling time are shown.

Wounded leaf tissue, sampled 6 and 2 mm from a necrotic leaflet tip, looked similar to healthy tissue except for a slight accumulation of spherical protein bodies. No cubical crystals were observed. A naturally senescent, chlorotic leaf from a 2-month old tomato plant was also fixed and observed by light microscopy. Although the leaf tissue had the general appearance of the coronatine-treated tissue at 12 days, no spherical or cubical protein bodies were observed.

Proteinase inhibitors and *P. syringae* pv. *tomato*.

The effect of proteinase inhibitors on the growth of *P. s.* pv. *tomato* was tested by determining the growth rate of PT23.2 in H_2O containing healthy or chlorotic tomato leaf tissue (Fig. 9). Interestingly, the growth rate of bacteria in H_2O containing chlorotic tissue was greater than the growth rate in H_2O containing healthy tissue. To further determine whether a purified chymotrypsin inhibitor adversely affects the growth of *P.* pv. *tomato*, MG broth was amended with varying concentrations of inhibitor. Growth of PT23.2 and PT23.21 was not significantly enhanced nor reduced at any concentration tested (data not shown).

DISCUSSION

Many chlorosis-inducing phytotoxins, including tagetitoxin, cercosporin, tentoxin, and victorin, act by disrupting plastid structure (Lukens and Durbin 1985; Steinkamp et al. 1981; Klotz 1988; Strobel et al. 1972). Coronatine-treated tissue, however, maintains a relatively normal ultrastructure and instead actively accumulates relatively high levels of proteinase inhibitors. Although coronatine was previously shown to induce hypertrophy in potato tubers (Sakai 1980) and tobacco leaves (Kenyon and Turner 1990), we observed no differences in cell dimensions or numbers in coronatine-treated and -untreated tomato leaves. Furthermore, all cells in coronatine-treated tissue were intact, indicating that this toxin does not disrupt the structural integrity of plant cells.

The chloroplasts in coronatine-treated tissue were intact and well-structured, suggesting that they were still functional. This hypothesis is supported by Kenyon and Turner's (1990) observations that photosynthesis and chlorophyll content declined in coronatine-treated tissue, but the rate of photosynthesis per unit of chlorophyll remained constant, suggesting a functional photosynthetic system. Interestingly, we noted that coronatine-treated chloroplasts were significantly smaller and darker-staining than chloroplasts in healthy tissue. This could be explained by at least two possibilities: (i) starvation of the cell due to reduced photosynthesis may exhaust certain metabolites in the chloroplast and result in shrinkage; or (ii) coronatine-treated tissues may be more permeable to the fixatives and stains

Table 2. Systemic effects of coronatine treatment on tomato leaf tissue

Sampling sites[a]	Chlorosis	Chloroplasts[b]	Epidermal cell walls[c]	Crystals[d]	SPBs[d]
Treated leaflet	Intense	Shrunken, descended	Thickened	46	2,900
Adjacent leaflet	Slight	Shrunken, descended	Thickened	22	1,070
Opposite leaf	None	Very slightly shrunken	Normal	0	420
Upper leaf 1	None	Normal	Normal	0	320
Upper leaf 2	None	Normal	Normal	0	200
Control	None	Normal	Normal	0	0

[a] Sampling sites consisted of the following locations on a plant inoculated with 125 ng of coronatine. Treated leaflet = terminal leaflet on the second primary leaf, treated with coronatine; Adjacent leaflet = leaflet adjacent to the treated leaflet; opposite leaf = first primary leaf, directly opposite the treated leaf; upper leaf 1 = third primary leaf, directly above the treated leaf; upper leaf 2 = fourth primary leaf; control = second primary leaf on a separate plant inoculated with water only. The experiment was repeated with similar results.

[b] Chloroplasts were categorized as being normal in size, very slightly shrunken, or significantly smaller than normal and located near the bottom of the palisade mesophyll cells (shrunken and descended).

[c] Upper epidermal cell walls were considered thickened if they were at least 2 times thicker than those present in control tissue when viewed by light microscopy.

[d] Average numbers of crystals and spherical protein bodies (SPBs) that were counted in 100 palisade mesophyll cells.

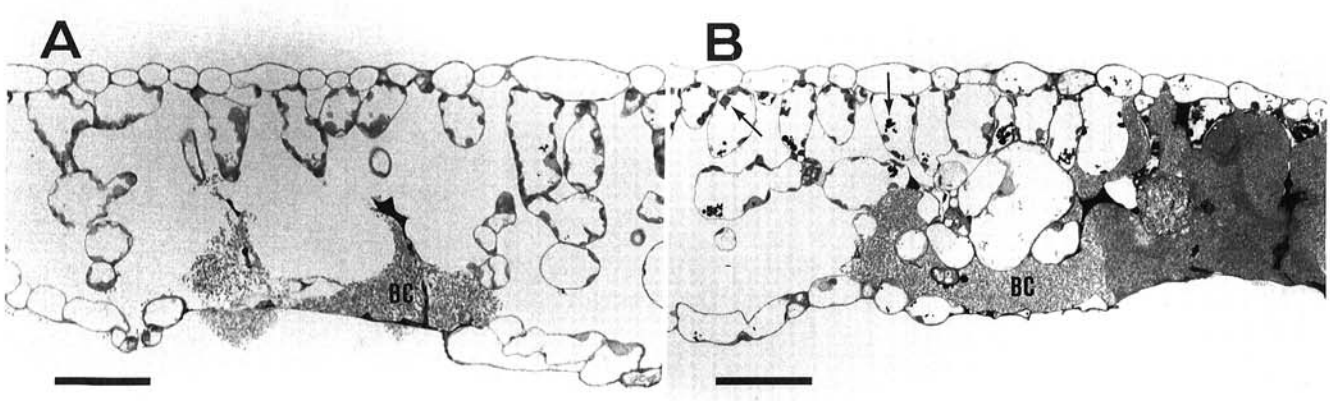

Fig. 7. Cross section of tomato leaf tissue inoculated with Cor^+ and Cor^- strains of *Pseudomonas syringae* pv. *tomato*. **A,** Lesion produced by the Cor^- PT23.21. Cells surrounding the relatively small bacterial colony (BC) show normal morphology. **B,** Lesion induced by the Cor^+ PT23.2. Note the shrunken chloroplasts and accumulation of protein bodies (arrows) in cells adjacent to the relatively large bacterial mass (BC). Bar = 50 μm.

used in tissue preparation.

Given the lack of chlorophyll and subsequent reduced photosynthetic ability in chlorotic tissue, the occurrence of starch deposits in chloroplasts 12 days after coronatine treatment was surprising. Since chloroplasts lacking chlorophyll are unlikely to have an energy surplus, this suggests an inability of chloroplasts to metabolize stored starch deposits. Coronatine may therefore block catabolic pathways within the chloroplasts.

Large, proteinaceous, cubical crystals were consistently observed in all coronatine-treated tissues. Other researchers have noted similar proteinaceous crystals in excised, washed, beet roots; excised, starved, tobacco leaves; or commercially grown or excised tomato leaves (Steinkamp et al. 1981; Ragetli et al. 1970; Shepardson 1982; Akers and Hoff 1980). Akers and Hoff (1980) observed a strong (r = 0.999) correlation between the volume of the cubical crystals in tomato leaf parenchyma cells and chymopapain inhibitor activity, while similar crystals were found in potato tubers and were inhibitory to chymopapain, papain, and ficin (Rodis and Hoff 1984). Since the crystals previously described by Akers and Hoff are identical in appearance and location to those observed in our study, and since we observed a high concentration of chymopapain inhibitor activity in coronatine-treated leaf tissue, it is highly likely that the cubical crystals observed in the present study are deposits of chymopapain inhibitors.

Spherical protein bodies were also consistently seen in all coronatine-treated tissue. Identical protein bodies were previously observed in light-stressed tomato mesophyll cells and were shown to be accumulations of chymotrypsin inhibitor I protein (Shumway et al. 1970; Shumway et al. 1976). The structural similarity between the spherical protein bodies described in the present study and those previously reported (Shumway et al. 1976) and the large amount of chymotrypsin inhibitor activity observed in coronatine-treated tissue strongly suggest that the spherical protein bodies observed in the present study are chymotrypsin inhibitors. Immunocytochemical studies to positively identify the spherical bodies and the cubical crystals are currently under way.

The massive accumulation of proteinase inhibitors in tomato leaves exposed to coronatine can be related to several other studies. Pautot et al. (1991) demonstrated the accumulation of chymotrypsin inhibitor I and II mRNAs in tomato leaves infected with *P. s.* pv. *tomato* PT11, a producer of coronafacoyl compounds (Palmer and Bender, unpublished observations). Feys et al. (1994) showed that chymotrypsin inhibitors accumulated in tomato leaflets after exposure to coronatine. Furthermore, airborne methyl jasmonate, a compound with marked structural similarity to coronatine (Fig. 1), caused the accumulation of chymotrypsin and papain inhibitors in exposed tomato leaves (Farmer and Ryan 1990; Bolter 1993).

Several recent studies have demonstrated similarities between the biological activities of coronatine and methyl jasmonate (Weiler et al. 1994; Feys et al. 1994). The present study indicates that the compounds do not induce identical responses in tomato leaf tissue. The application of methyl jasmonate did not result in the leaf chlorosis that is typical of coronatine, nor did it induce shrinkage of chloroplasts or thickening of epidermal cell walls. Since coronafacic acid induced symptoms similar to methyl jasmonate in the present study and is more closely related to the structure of methyl jasmonate than coronatine (Fig. 1), it is tempting to speculate that the CFA moiety alone is responsible for the similar effects obtained with methyl jasmonate and coronatine. Further experiments using coronatine precursors and analogues are presently under way to determine which sites on the coronatine molecule are responsible for the different biological responses observed in the present study.

Although most of the effects attributed to coronatine were localized in or near the chlorotic tissue, the accumulation of spherical protein bodies was systemic. Chymotrypsin inhibitors were previously shown to be systemically distributed in tomato plants after severe mechanical injury; the accumulation of inhibitor was mediated by spread of a proteinase inhibitor-inducing factor (PIIF) from the wounded site (reviewed in Ryan 1992). Since methyl jasmonate is a possible PIIF (Ryan 1992), and because coronatine and methyl jasmonate both induce proteinase inhibitors, it is tempting to speculate that coronatine itself might induce systemic accumulation of proteinase inhibitors in toxin-treated tomato plants.

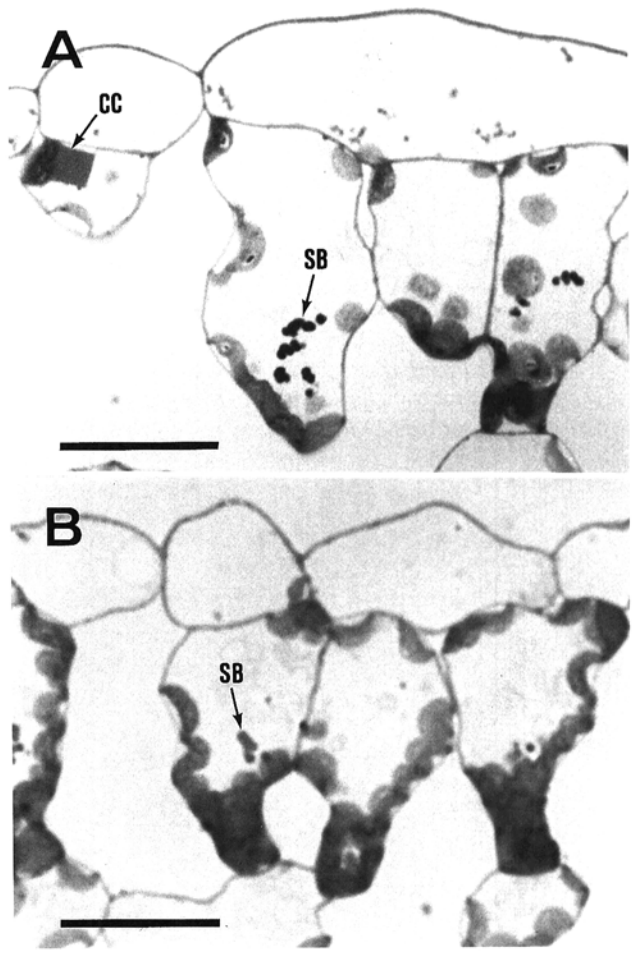

Fig. 8. Tissue treated with analogues of coronatine. **A,** Methyl jasmonate-treated tissue showing cubical crystals (CC) and spherical protein bodies (SB). **B,** Tissue treated with coronafacic acid showing a slight accumulation of spherical protein bodies (SB). Cell walls and chloroplasts appear normal in both treatments. Bar = 25 µm.

Interestingly, the plant defense response triggered by coronatine does not slow pathogen growth and multiplication. Several lines of evidence indicate that the accumulation of proteinase inhibitors has little effect on lesion dynamics. Primarily, both Cor$^+$ and Cor$^-$ strains grew and multiplied in tomato leaf tissue, even though the Cor$^+$ strain induced a massive accumulation of proteinase inhibitors. Secondly, the in vitro study demonstrated that the Cor$^+$ PT23.2 grew better in solutions containing chlorotic tissue than in solutions containing healthy tissue, despite the accumulation of inhibitors in the former. Furthermore, chymotrypsin inhibitor did not impact growth of PT23.2 or PT23.21 in vitro. Although proteinase inhibitors from cabbage foliage were shown to have antifungal activity (Lorito et al. 1994), and a TMV-induced proteinase inhibitor was shown to inhibit microbial proteinases (Geoffroy et al. 1990), the proteinase inhibitors induced by coronatine do not slow the growth of *P. s.* pv. *tomato*.

Precisely how coronatine acts as a virulence factor remains a mystery. Although coronatine does not destroy organelles or radically disrupt membrane systems, the Cor$^+$ PT23.2 was more virulent than the Cor$^-$ PT23.21 (consistent with Bender et al. 1987). Mittal and Davis (1995) recently demonstrated that coronatine suppressed the induction of phenylalanine ammonia lyase and other defense genes in *Arabidopsis*; however, our results and those of Feys et al. (1994) show that coronatine-treated tomatoes accumulate proteinase inhibitors, compounds known to protect plants from insect damage. Some of these disparate observations may reflect the host and tissue specificity of coronatine.

Since coronatine does not cause widespread cellular destruction, it may instead enhance virulence by inducing low-level leakage of cellular metabolites. This hypothesis is supported by observations that coronatine induces electrolyte leakage from Italian ryegrass leaves and may stimulate membrane-bound ATPases (Sakai et al. 1979 and 1984). Nutrient leakage induced by coronatine may play an important role in pathogen spread. Many leaf-spotting bacterial pathogens tend to be localized within the leaf lamina as an aggregated mass of bacterial cells and slime (Williams and Keen 1967; Creager and Matherly 1961), and we have observed that coronatine production significantly increases colonization of tomato leaves by *P. syringae* pv. *tomato*. The larger bacterial cell mass would undoubtedly contact more stomata and would potentially secrete more bacterial ooze to the leaf exterior, thereby facilitating dissemination of the pathogen.

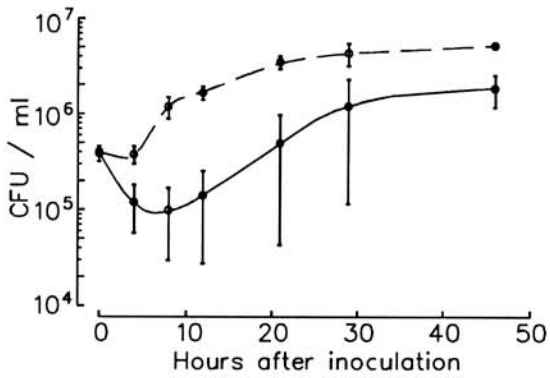

Fig. 9. Growth of PT23.2 on coronatine-treated chlorotic tomato leaf tissue (dashed line) and water-treated healthy tissue (solid line). Populations of bacteria growing on 4 cm^2 crushed leaf tissue suspended in 50 ml of H_2O were monitored at various time points by dilution plating. Error bars represent standard error.

The precise mode of action for coronatine will only be elucidated by locating the putative receptor for the toxin within the plant. Experiments of this nature are currently under way and will provide exciting new insights into how the coronatine molecule perturbs host plant metabolism.

METHODS AND MATERIALS

Plants.

Lycopersicon esculentum Mill. cv. Marglobe was used in all experiments. Plants were grown from seed in a peat:soil mix in 10-cm-diameter plastic pots and maintained at 25°C, 40 to 70% RH. A 12-h photoperiod was provided with four General Electric F40PL/AQ broad-spectrum fluorescent bulbs located 30 cm above the soil level. Plants were approximately 6 weeks old at the time of inoculation.

Preparation of treatments.

Coronatine was prepared by the method of Palmer and Bender (1993) and was further purified by thin-layer chromatography on 0.25-mm silica plates in isopropanol/acetic acid/H_2O:ethyl acetate 8:1:1:390. Coronafacic acid was prepared by hydrolyzing coronatine in 6 M HCl at 100°C for 24 h and recovering the organic acid phase (this process was repeated to ensure complete hydrolysis of coronatine). Methyl jasmonate was obtained from Bedoukian Research Inc, Danbury, CT. *Pseudomonas syringae* pv. *tomato* strains PT23.2 (Cor$^+$, Bender et al. 1987) and PT23.21 (Cor$^-$, Bender et al. 1987) were grown approximately 48 h on mannitol-glutamate (MG) medium (Keane et al 1970). Immediately prior to inoculation, a loopful of cells was suspended in sterile H_2O, centrifuged and the pellet resuspended in sterile H_2O at approximately 1×10^9 CFU/ml.

Inoculation and tissue sampling.

In all treatments, the underside of the second primary leaf was inoculated within 3 days after full expansion. Coronatine was applied at 125 ng, coronafacic acid at 75 ng (equimolar to coronatine), and methyl jasmonate was applied at 125, 1,250, 12,500, and 125,000 ng. These compounds were suspended in water and applied as small (less than 5 µl) droplets on the undersides of tomato leaves. Plants inoculated with the volatile methyl jasmonate were placed in a room separate from all other plants. Plants were wounded by scorching a leaflet tip with an open flame. *P. s.* pv. *tomato* cells were applied by gently rolling a cotton swab soaked in a bacterial suspension along the underside of a leaflet. Control sites were inoculated with sterile distilled H_2O applied as a 2.5 µl drop (in experiments where coronatine, coronafacic acid, and MeJas were applied) or by gently rolling a H_2O-soaked cotton swab along the leaflet (in experiments where bacterial suspensions were applied). All treatments were examined in at least three separate experiments. After an appropriate time period (8 days with the exception of the time course experiment), leaf tissue was excised and processed. At least five sites per leaf were sampled in each treatment.

Tissue processing and microscopy.

Immediately after excision, leaf tissue was infiltrated with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Tissue was fixed for 2 h at 24°C, rinsed in buffer, and postfixed in 1% osmium tetroxide for 2 h. After dehydration in a graded ethanol series, the tissue was embedded in Spurr's low viscosity resin using propylene oxide as a transitional solvent. Thick and thin sections were prepared with a Sorvall MT-2 ultramicrotome. Thick sections (1 μm) were stained with Mallory's stain and viewed with a Nikon Labophot-2 microscope. Thin sections (140 nm) were stained with uranyl acetate and lead citrate and viewed with a JEOL-100CX scanning transmission electron microscope at 60 kV. SEM samples were fixed similarly, critical-point dried after dehydration, Au-Pd sputter-coated, and viewed with a JSM-35U scanning electron microscope at 25 kV.

Chlorophyll assay.

Leaf chlorophyll content was measured by extracting crushed leaves with ice-cold ethyl acetate/acetone (1:1, v/v). The extracts were then diluted (1:33) in the same solvent, and chlorophylls a and b were estimated by measuring the absorbance at A_{665} and A_{649} nm, respectively.

Proteinase inhibitor assays.

Leaf extracts were tested for proteinase inhibition using the method of Akers and Hoff (1980). Chymopapain (EC 3.4.4.11) and chymotrypsin (EC 3.4.22.6) were obtained from Sigma Chemical Co, St. Louis, MO. Briefly, filter paper strips were soaked in leaf sap from approximately 4 cm^2 of healthy or treated tomato tissue and placed on casein-agar medium (1% casein, 1.4% agar, 3 mM $MgCl_2$, 2 mM Na_2EDTA, 5 mM dithiothreitol) for 20 min. The strips were then removed, and a second set of filter paper strips soaked in an enzyme dilution series was placed at right angles to the first strips. After 20 min, the enzyme strips were removed and the plate was allowed to develop at 50°C for 4 h. Inhibitor activity was visible as constrictions or breaks in the white lines of precipitate formed by enzyme activity. Activity was reported as the maximal concentration of enzyme completely inhibited by the plant extract.

The effect of a chymotrypsin inhibitor on growth of *P. s.* pv. *tomato* PT23.2 and PT23.21 was assayed as follows. Aliquots (10 ml) of MG medium, supplemented with 0, 0.1, 1, 10, and 100 mg of chymotrypsin inhibitor (Sigma Chemical Co., St. Louis, MO) per liter, were inoculated with 10 μl of stationary-phase PT23.2 or PT23.21 broth cultures. Bacteria were further incubated at 28°C on a rotary shaker at 280 RPM and turbidity was observed at 24 and 48 h.

PT23.2 growth rate assay.

A comparison of growth rates for PT23.2 on chlorotic and healthy tissue was made by preparing duplicate flasks containing 50 ml H_2O, 4 cm^2 crushed chlorotic or healthy leaf tissue, and 25 μg/ml rifampicin. Flasks were inoculated with 10 μl of PT23.2 grown to stationary phase, and bacterial populations were determined by dilution plating at various intervals for 46 h.

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