# Fluorescing Materials Associated with Vein Blackening and Necrosis in Leaves of Black Rot-Resistant Cabbage

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ABSTRACT

The veins and hydathodes of resistant and susceptible cabbage leaves were inoculated with aqueous suspensions of *Xanthomonas campestris*. An accumulation of a blue-green fluorescing material ($E_{max}$ = 500 to 510 nm) in the lumen of xylem vessel elements prior to vein darkening was observed only in lines containing the *f* gene for resistance to *X. campestris*. The fluorescence within the lumen disappeared as the infected vessels darkened and fluorescence appeared in the parenchymatous tissues of the hydathodes. In leaves of susceptible plants, a red-brown fluorescence which was unstable to ultraviolet light accumulated in the cell walls of the bundle sheath sclerenchyma.

Following infiltration of leaf panels with $10^8$ cells/ml of *X. campestris*, the rates of bacterial multiplication and lesion development were similar in resistant and susceptible plants, indicating that the intercostal parenchyma is not directly involved in resistance. The necrotic responses of cabbage leaf parenchyma to *X. campestris* were identical to those elicited by incompatible or heterologous plant pathogens, *X. malvacearum, X. vasculorum, Pseudomonas syringae,* and *P. solanacearum*. A blue-green fluorescence could be observed in the bundle sheath of veins surrounding the necrotic panels induced by both homologous and heterologous organisms. Only slightly more fluorescence appeared in resistant than in susceptible plants.

A histological comparison of hydathodes infected with *X. campestris* from resistant and susceptible plants showed extensive maceration of xylem vessel terminals and surrounding parenchyma in susceptible plants, whereas the corresponding tissues in resistant plants were not macerated.

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Cabbage plants resistant to black rot are characterized by the absence of chlorotic v-shaped lesions containing blackened veins and by the absence of systemic invasion of the xylem by *Xanthomonas campestris* (25). In resistant plants, the bacteria were restricted to necrotic areas comprising marginal intercostal regions or to necrotic hydathodes. However, the presence of these same necrotic reactions on susceptible plants gave reason to question their involvement in the resistance mechanism.

Although the formation of spreading lesions on resistant plants after inoculation of injured vein endings implicated the hydathode as the site of resistance, an induced inhibition of lesion growth and bacterial multiplication has been demonstrated within the major veins (21). In resistant genotypes, the hydathode, the vein, and the intercostal parenchyma have all been implicated as possible sites of black rot resistance (21); these tissues were examined cytologically in relation to bacterial multiplication.

A preliminary examination of marginal necrotic lesions on field-inoculated cabbages showed that fluorescent material accumulated in the bundle sheath of veins surrounding the marginal necrotic panels. This suggested possible changes in aromatic metabolism of the leaf after infection. Since changes in aromatic compounds after vascular infection by a number of pathogens have been demonstrated both by extraction procedures (8, 12, 17, 19) and by fluorescence microscopy (4, 9, 10), the evolution of fluorescence in black rot-infected cabbage was examined in more detail. The buildup of fluorescing materials in vascular bundles of resistant and susceptible genotypes was examined microscopically following inoculation of the leaves by several methods.

MATERIALS AND METHODS.—Black rot-resistant and -susceptible cabbage plants, *Brassica oleracea* L. var. *capitata* L., were grown and the inocula of *Xanthomonas campestris*, isolate B-87 S2, prepared as described previously (21). Seven- to 12-week-old plants were inoculated either at guttating hydathodes, at injured vein endings (21), or by injection of bacterial suspensions into the leaf parenchyma.

Because of our difficulty in obtaining uniform results by inoculating leaves via the hydathodes (21) to determine bacterial multiplication, multiplication of *X. campestris* in leaf parenchyma was examined. About 25 panels on fully expanded leaves on each of six resistant and six susceptible plants were infiltrated with $10^8$ cells/ml of *X. campestris* by means of a hypodermic syringe equipped with a 26-gauge needle. Bacteria at the edges of infiltrated panels were sampled by punching out 3.5-mm discs of the veins limiting the panels. At each sampling time, one panel/plant was sampled in the center and at the edge. Bacterial numbers were determined by dilution plating.

The response of resistant and susceptible leaf parenchyma to various concentrations of *X.*

*campestris* was compared to the reactions to an avirulent isolate of *X. campestris* (B-87 S1), *Pseudomonas solanacearum, P. syringae, X. malvacearum, X. vasculorum,* and a pectolytic *Pseudomonas* sp. from rotting cabbage heads. Cells were suspended in sterile distilled water containing 0.5% $MgSO_4$.

Fluorescence in cabbage tissue was observed in fresh freehand sections with a Zeiss Universal microscope using epi-illumination provided by a stabilized high-pressure mercury lamp (Osram, HBO-100 W/2), supplemented with the excitation filters BG 38 and BG 3 (Schott, Mainz). Fluorescence emission was selected with the barrier filters 41 and 44, resulting in the transmission of the light above 440 nm. A Zeiss interference wedge monochrometer was used to determine fluorescence emission spectra. Relative fluorescence intensities were measured at wavelength intervals of 10 nm with a Photovolt Model 520 SP photometer (Photovolt Corp., New York, N.Y.) and recorded with a Heath Model EU-208 servo-recorder (Heath Co., Benton Harbor, Mich.). Photomicrographs of fluorescent tissue sections were taken on polaroid film (Type 107).

To obtain histological evidence of the nature of limitation of *X. campestris* in resistant plants, lesions were fixed in Formalin-acetic acid-alcohol (FAA) 7 days after hydathode inoculation and embedded in paraffin (7). Unstained sections were observed for tissue darkening along the veins limiting the necrotic lesions and in darkened veins of susceptible type lesions. Sections were stained with thionin blue-orange G or with toluidine blue (7).

RESULTS.—Within the centers of intercostal panels of resistant and susceptible leaves infiltrated with $10^8$ cells/ml, bacteria grew rapidly for 48 to 60 hr, then growth stopped abruptly as the infiltrated tissues collapsed (Fig. 1). In resistant leaves, the bacterial populations started to decrease immediately following the collapse of the panels, whereas in susceptible leaves they remained close to the maximum level for 4 days before decreasing to zero in the necrotic panels around the 12th day after inoculation (Fig. 1). In three experiments, the maximum populations reached ranged from 1.1 to $3.3 \times 10^6$ cells/resistant leaf disc and from 3 to $8 \times 10^6$ cells/susceptible leaf disc.

At the edges of the necrotic panels, the bacterial populations stayed near the maximum in both resistant and susceptible lines for over 20 days. Population levels at the panel margin, however, were consistently significantly (99 to 95% level) 3-5 times higher in susceptible than in resistant plants (Fig. 1).

The time at which leaf parenchyma injected with *X. campestris* developed symptoms was dependent on the inoculum dosage, but independent of the host genotypes at 20 and 28 C (Table 1). Lower inoculum levels were needed at 28 than at 20 C to elicit equivalent host responses. The time required for symptom appearance increased similarly with decreasing inoculum dose on the four cabbage lines tested. The minimum response times were 24 hr at 28 C and 48 hr at 20 C, and the lowest inoculum

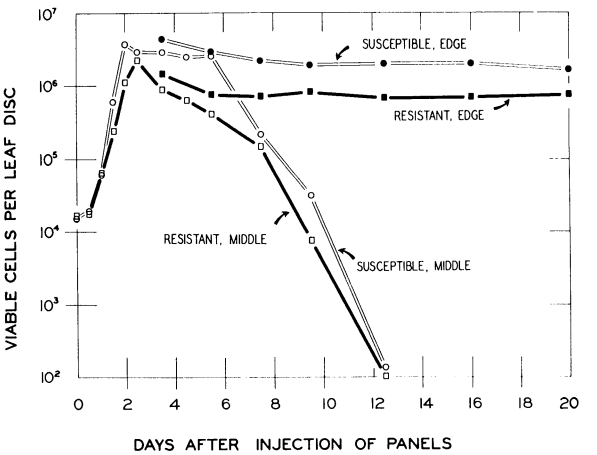


**Fig. 1.** Viable cells of *Xanthomonas campestris* in intercostal leaf parenchyma of resistant (Early Fuji inbred) and susceptible cabbage (Sanibel) 0 to 20 days after infiltration of $10^8$ cells/ml.

levels to elicit a response within 10 days were $10^{2.5}$ cells/ml at 28 C and $10^4$ cells/ml at 20 C. Occasionally, chlorotic black rot lesions developed from leaf panels injected with *X. campestris* on susceptible plants, but never on resistant plants.

When other plant-pathogenic bacteria were infiltrated into the leaf mesophyll tissues of two resistant and two susceptible lines at $10^9$ cells/ml, host responses were identical to those caused by the virulent isolate of *X. campestris*, with exception of the pectolytic *Pseudomonas* sp., which occasionally produced necrosis in part of the panels (Table 2). Necrotic reactions occurred in continuous dark as well as in the light. Although the host reactions appeared to be the same after infiltration of all organisms, heterologous bacteria failed to survive near the veins limiting the necrotic panels as well as the two isolates of *X. campestris* (Table 2).

After vein inoculation at 20 to 28 C in growth rooms and in greenhouses, qualitative differences could be observed in the evolution of fluorescent materials in resistant and susceptible plants. In veins of resistant plants, vein blackening was preceded by the increase of blue-green fluorescence in the lumens of the xylem vessel elements (Fig. 2). Viewed in transmitted visible light, the fluorescing vessel elements had a red-brown to orange appearance. As the infected xylem vessel elements of resistant plants turned dark, the fluorescence in the lumens of vessel elements decreased and vanished completely at the point when vein blackening became macroscopically visible. In susceptible plants, no fluorescence could be detected in the xylem or xylem parenchyma (Fig. 2).

As the veins of susceptible plants turned black, a compound with red-brown fluorescence accumulated in the thickened cell walls of the bundle sheath sclerenchyma (Fig. 3). This compound was distinct from the compound with bright blue-green fluorescence ($E_{max}$ 500 to 510 nm) accumulating in

TABLE 1. Symptom appearance following infiltration of various concentrations of *Xanthomonas campestris* into leaf panels of resistant and susceptible cabbage plants

| Host line | Inoculum viable cells/ml (Log base 10) | Symptoms observed[a] At 20 C — Time after infiltration (hr) 24 | 48 | 72 | 120 | 240 | At 28 C — Time after infiltration (hr) 24 | 48 | 72 | 120 | 240 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sanibel (susceptible) | 2.5 | | | | | | | | | | (P) |
| | 4.0 | | | | | (P) | | | | | C |
| | 5.5 | | | | C | P | | | | C | N |
| | 7.0 | | | | S | –N | | –C | S | N | N |
| | 8.5 | | | C | N | N | C | S | N | N | N |
| | 10.0 | | C | N | N | N | S | S | N | N | N |
| Badger Inbred 3 (susceptible) | 2.5 | | | | | | | | | | (P) |
| | 4.0 | | | | | (P) | | | | | C |
| | 5.5 | | | | C | CP | | | | S | N |
| | 7.0 | | | | S | –N | | | S | N | N |
| | 8.5 | | | C | N | N | | S | N | N | N |
| | 10.0 | | C | N | N | N | S | S | N | N | N |
| Early Fuji (resistant) | 2.5 | | | | | | | | | | |
| | 4.0 | | | | | (P) | | | | –C | C |
| | 5.5 | | | | C | CP | | | | S | N |
| | 7.0 | | | | C | –N | | C | S | S | N |
| | 8.5 | | | N | N | N | C | S | N | N | N |
| | 10.0 | | C | N | N | N | S | S | N | N | N |
| $F_3$ line (resistant) | 2.5 | | | | | | | | | | (P) |
| | 4.0 | | | | | (P) | | | | | CP |
| | 5.5 | | | | | P | | | | S | N |
| | 7.0 | | | | C | CP | | C | S | N | N |
| | 8.5 | | | C | C | N | C | S | N | N | N |
| | 10.0 | | C | N | N | N | S | N | N | N | N |

[a] P = pinpoint black lesions throughout the infiltrated panels; C = chlorosis; S = panel collapsed without browning; N = necrosis of infiltrated panels; – = a given symptom was only slight; ( ) = a given symptom appeared only in part of the panels.

TABLE 2. Reaction of cabbage leaf parenchyma tissue following infiltration of ca. $10^9$ cells/ml of several plant pathogenic bacteria and bacterial populations at the edge of the necrotic lesions

| Organism | Viable cells/ leaf disc[a] | Symptoms observed — Hr after infiltration 12 | 36 | 60 | 84 | 168 |
|---|---|---|---|---|---|---|
| *Xanthomonas campestris* (virulent) | 980,000 | (C)[b] | S | N | N | N |
| *X. campestris* (avirulent) | 600,000 | (C) | S | N | N | N |
| *X. vasculorum* | 22,000 | (C) | CS | N | N | N |
| *X. malvacearum* | 40,000 | (C) | CS | N | N | N |
| *Pseudomonas syringae* (bean isolate) | 11 | (C) | CS | N | N | N |
| *P. syringae* (peach isolate) | 114 | C | CS | N | N | N |
| *P. solanacearum*, $B_1$ | 0 | (C) | S | N | N | N |
| *Pseudomonas* sp. (pectolytic) | 0 | (C)[c] | (C) | (CN) | (CN) | (CN) |

[a] Samples taken at the edge of the necrotic panels 152 hr after infiltration.

[b] Symptoms observed: C = chlorosis; S = panels collapsed without browning; N = necrosis of infiltrated panels; ( ) = a given symptom appeared only in part of the panels.

[c] Symptoms were erratic with this isolate.

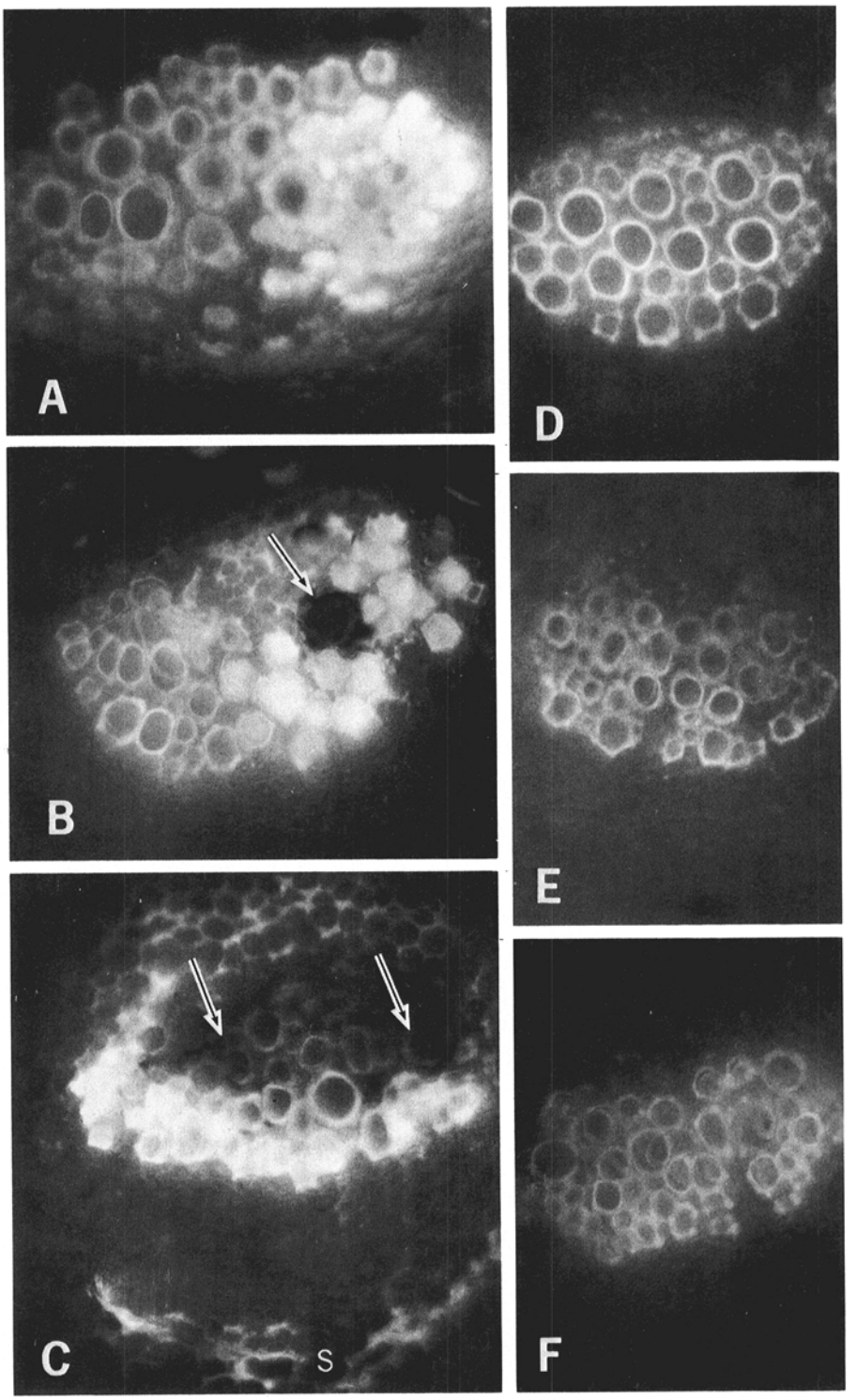


**Fig. 2.** Fluorescence in fresh cross sections of vascular bundles of cabbage leaves infected with *Xanthomonas campestris* at different stages of disease development. **A, B, C, D)** Resistant plants: **A)** blue-green fluorescence first visible in xylem vessel elements showing a faint red-brown discoloration under visible light (× 410); **B)** (arrow) fluorescence in partially darkened bundle (× 360); **C)** fluorescence at point of macroscopic vein blackening (× 310); note loss of fluorescence in blackened portions of veins (arrows) and fluorescence in bundle sheath (S); **D)** noninoculated control (× 360). **E, F)** Susceptible plants: **E)** fluorescence absent in partially darkened bundle (× 310); **F)** noninoculated control (× 310).

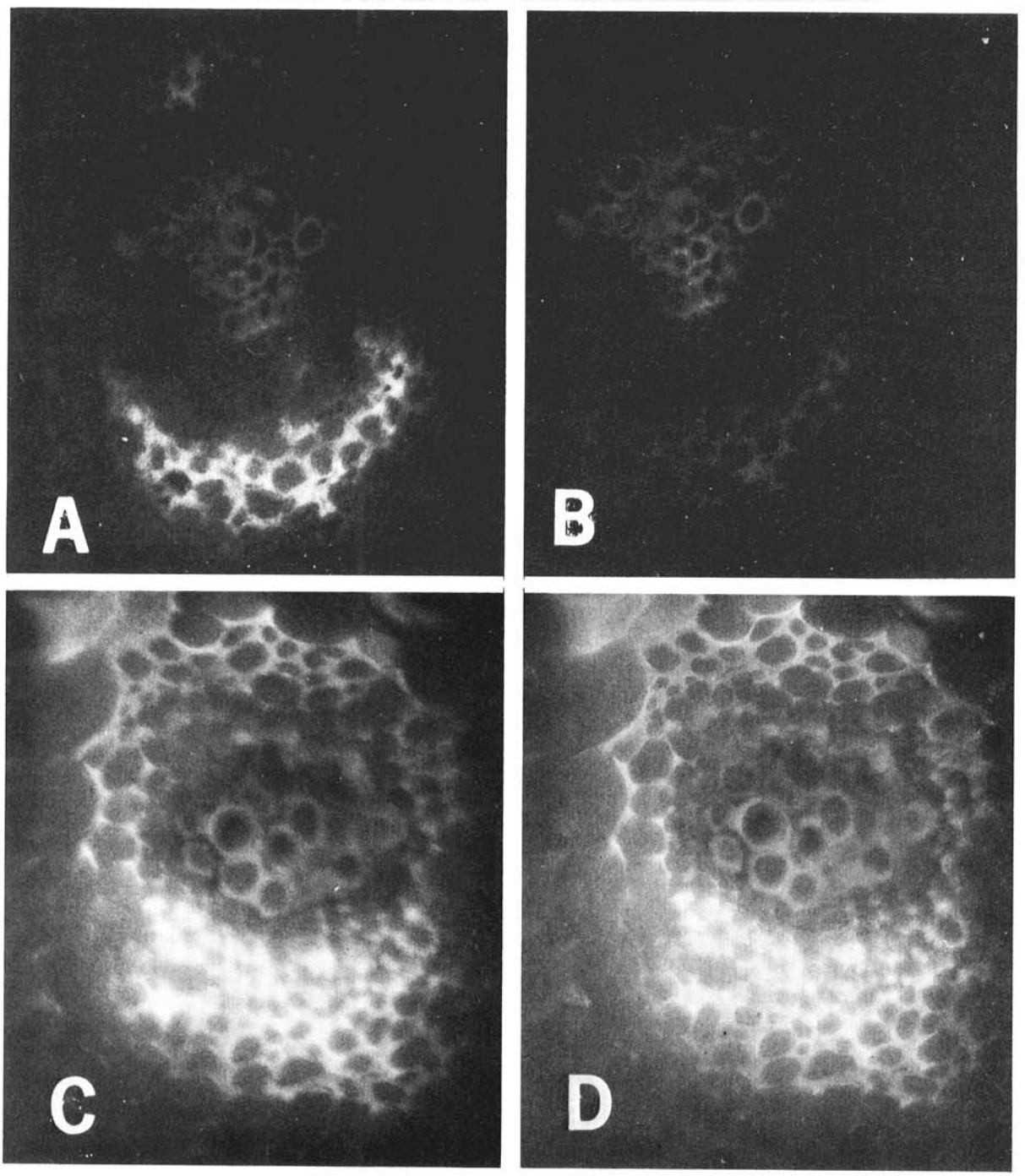


**Fig. 3.** Sequential photomicrography of cross sections of fluorescing vascular bundles of resistant and susceptible cabbage leaves inoculated at injured vein endings with *Xanthomonas campestris* (× 300). **A, B)** Section from a susceptible leaf: **A)** film exposed during the first 120 sec; **B)** during the second 120 sec of ultraviolet illumination. **C, D)** Section from a resistant leaf: **C)** film exposed during the first 90 sec; **D)** film exposed from the 300th-390th sec of ultraviolet illumination. Note that the fluorescing compound from susceptible plants broke down under ultraviolet exposure, but the fluorescing compound in resistant plants was stable under ultraviolet light.

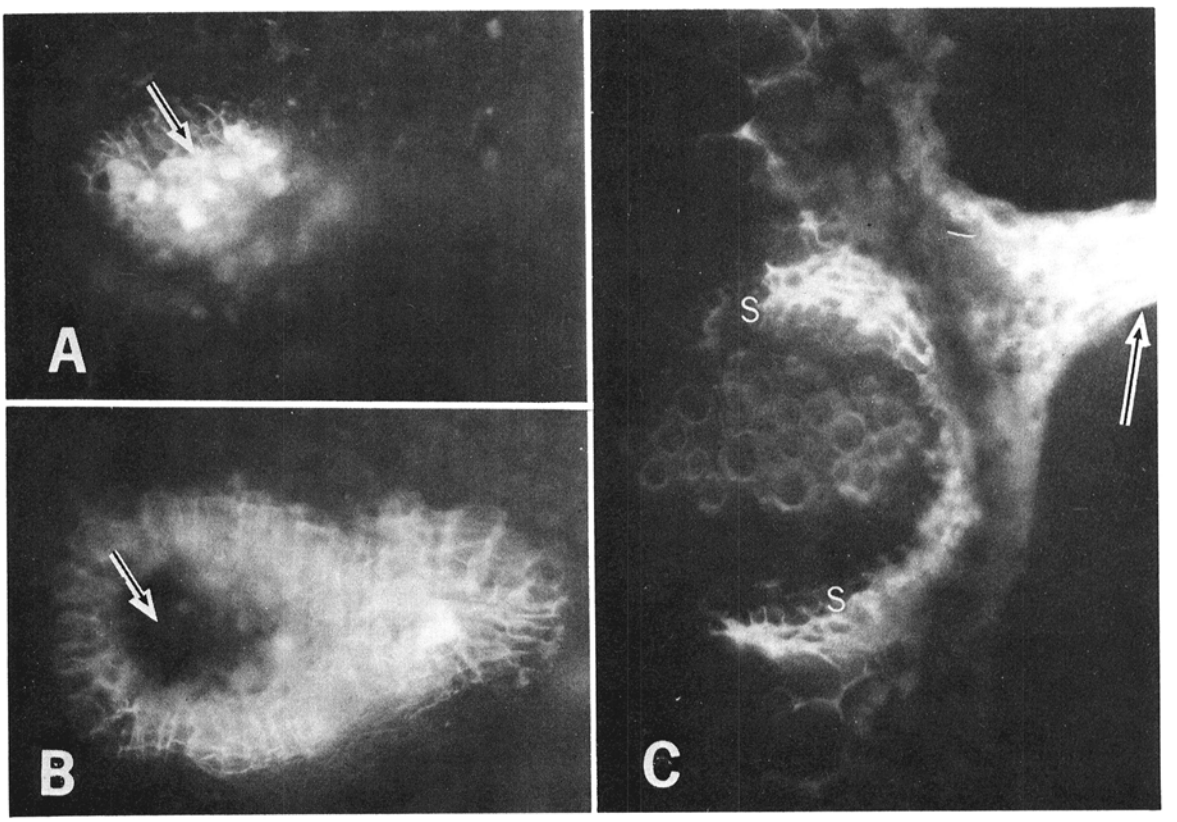


**Fig. 4.** Fluorescence following inoculation of cabbage leaves with *Xanthomonas campestris* through the hydathodes (A, B) and by infiltration of leaf panels (C). **A)** Fluorescence in fresh cross sections of xylem vessel terminals (arrow) in hydathodes of resistant plants prior to darkening of xylem vessels (× 420). **B)** Fluorescence in hydathode parenchyma around darkened xylem vessel terminals (× 420); note absence of fluorescence in dark xylem vessel terminals (arrow). **C)** Fluorescence in the bundle sheath (S) of a vein delimiting a necrotic panel (arrow) (× 240); resistant and susceptible plants showed similar fluorescence around necrotic panels.

the corresponding tissue of resistant plants. The blue-green fluorescence was stable under ultraviolet light, whereas the red-brown fluorescence vanished rapidly. The half-life of the material with the red-brown fluorescence under the ultraviolet intensity used was ca. 19 sec. The breakdown of red-brown fluorescence under ultraviolet illumination could be documented by the taking of serial photographs of the same sections as it is being irradiated (Fig. 3).

In the hydathodes of resistant plants, as in the major veins, a blue-green fluorescence preceded the darkening of the vein terminals. The vein darkening at the hydathode usually extended several millimeters beyond the necrotic portion of the hydathode without being visible externally. Again, fluorescence was most intense in the xylem vessels in which a faint reddish color appeared in visible light and fluorescence decreased as the vessels darkened. Concomitant with the decrease of fluorescence in darkening xylem vessel terminals, a fluorescence began to appear in the surrounding parenchyma cells (Fig. 4). The sequence from the initial appearance of fluorescence in a few xylem vessel elements to the complete blackening of the entire vascular bundle was condensed over 1 to 2 mm at the hydathode, whereas in the major veins it extended over several centimeters (Table 3).

Fluorescence in a resistant $F_3$ line of cabbage derived from a cross between resistant Early Fuji and Badger Inbred 3 (BI-3) was identical to that on the resistant Early Fuji. The susceptible BI-3 showed fluorescence similar to that of the susceptible cultivar Sanibel used throughout this study.

At the edges of infiltrated leaf panels of both resistant and susceptible plants, a blue-green fluorescence became visible in the cell walls of the bundle sheaths and in the intercellular spaces of the bundle sheath extensions (Fig. 4). The exact quantitation of this fluorescence was impossible due to the wide variation in fluorescence intensities and to the irregular areas which were fluorescing. A red-brown fluorescence, similar to that observed in the bundle sheath of blackened veins of susceptible plants (Fig. 3), appeared erratically in both lines within the intercellular spaces of the bundle sheath extensions, in particular close to the epidermis.

TABLE 3. Fluorescence in vascular bundles of resistant and susceptible cabbage leaves 6 to 8 days after vein inoculation with *Xanthomonas campestris*

| Color of vascular bundle | Length of vascular discoloration[a] | Color of fluorescence | | | |
|---|---|---|---|---|---|
| | | In xylem vessel lumen[b] | | In bundle sheath | |
| | | Resistant | Susceptible | Resistant | Susceptible |
| | *cm* | | | | |
| Black | 0 | None | None | Blue-green | Red-brown |
| Partially black[c] | 0-3 | Blue-green | None | None | None |
| Faint red | 3-4 | Blue-green | None | None | None |
| Normal | 4 | None | None | None | None |

[a] Measured proximally from point of visible vein blackening.
[b] Including surrounding xylem parenchyma.
[c] Only few of the xylem vessels in vascular bundle darkened.

Fluorescence intensities were estimated subjectively, taking into consideration both intensities and areas of fluorescence and both the blue-green and red-brown fluorescence.

When bacteria were introduced into the leaf mesophyll, fluorescence was indistinguishable in resistant and susceptible plants, but it appeared more rapidly and more intensely in resistant plants (Table 4). Bacteria did not penetrate the xylem vessels surrounding the infiltrated panels on resistant plants, and penetration occurred in only 1 out of 48 panels examined from susceptible plants.

Histological examination of hydathodes infected with *X. campestris* from susceptible plants revealed extensive maceration of the xylem vessel terminals and the surrounding hydathode parenchyma. A gelatinous mass having the staining properties of the bacterial mucopolysaccharide (22) had coagulated and was visible in long strands in the distorted xylem vessels. At the edge of the macerated tissue, unmacerated cells were wedged apart by bacteria occupying the intercellular spaces.

In infected blackened hydathodes of resistant plants, only a few of the xylem vessel terminals were filled with a granular material which, in some cases, was embedded in a deeply staining matrix. Although the protoplasts of the parenchyma cells in the blackened hydathodes had collapsed, no morphological changes in the walls of the xylem vessel terminals and the surrounding parenchyma could be observed.

Where bacteria had invaded the intercellular spaces of the leaf mesophyll, in either resistant or susceptible plants, the spongy mesophyll was most severely disrupted. Xylem vessels in the veins surrounding these necrotic lesions were free of plugging and darkening; but in susceptible lesions, infected xylem vessels were extensively plugged and surrounded with a dark material (22).

TABLE 4. Accumulation of fluorescing materials around veins surrounding intercostal regions of cabbage leaves infiltrated with $10^8$ cells/ml of *Xanthomonas campestris*

| Hr after infiltration | Intensity of fluorescence[a] | | | |
|---|---|---|---|---|
| | In bundle sheath | | In bundle sheath extension | |
| | Resistant | Susceptible | Resistant | Susceptible |
| 36 | 0 | 0 | 0 | 0 |
| 60 | 0-1 | 0 | 0-1 | 0-1 |
| 84 | 1 | 0-1 | 0-1 | 0-1 |
| 108 | 1-2 | 0-1 | 1-2 | 0-1 |
| 132 | 1-2 | 0-1 | 1 | 0-1 |
| 180 | 1-3 | 1-2 | 1-2 | 1-2 |
| 228 | 2-3 | 1-2 | 1-2 | 1-2 |
| 300 | 2-3 | 1-2 | 1-2 | 1-2 |

[a] Fluorescence intensity was rated in fresh freehand sections of veins under a fluorescence microscope as follows: 0 = no recognizable increase in fluorescence; 1 = faint fluorescence; 2 = moderate fluorescence; 3 = bright fluorescence.

DISCUSSION.—The appearance of vein blackening following the introduction of *X. campestris* into the xylem of resistant and susceptible plants indicated that alterations occur in the normal aromatic metabolism of plants with both genotypes (18). The appearance of fluorescence prior to vein darkening in the xylem vessels of inoculated hydathodes and veins of resistant cabbages indicated that *X. campestris* was capable of inducing changes in the aromatic pathways in plants containing the *f* gene for black rot resistance (25) which do not occur in susceptible genotypes. Though the evidence presented in this paper is indirect, the inhibition of both bacterial multiplication and black rot lesion formation on resistant plants (21) may be mediated by these changes in aromatic metabolism. Rubin & Artsikhovskaya (18) showed that oxidation products of phloroglucinol formed during the necrotic response of cabbage to *Botrytis cinerea* might result in limitation of the fungus on resistant plants. Reducing agents interfered with resistance in tobacco to bacterial wilt (13).

The similarities in blue-green fluorescence in the infected hydathodes and veins of resistant plants indicated that the same changes in aromatic metabolism are induced in these two tissues. Likewise, the appearance of red-brown fluorescence in both tissues of susceptible plants implicated a common aromatic metabolism in infected veins and hydathodes.

The effectiveness of resistance in the hydathodes, when compared with major veins (21), may be due to the greater proportion of potentially reactive cells surrounding the xylem vessels in the hydathodes than in the veins. This proportion could be expected to follow closely the surface to volume ratio of a cylinder.

Whether compounds induced in resistant plants are capable of inhibiting the pathogen directly is not known. The absence of tissue maceration in resistant plants points to the possibility that the aromatic compounds confer resistance by inactivating macerating enzymes (8, 11, 17) or by altering the degradability of mechanical barriers in the xylem (1). *Xanthomonas campestris* produces pectin esterase and polygalacturonic acid *trans*-eliminase in culture (15), and the extensive maceration observed in hydathode regions of susceptible plants and the formation of lysogenic cavities in infected veins (14, 16, 20, 22) indicated that these enzymes are active in pathogenesis. This lack of tissue maceration in resistant cabbage is similar to that described for cotton resistant to *X. malvacearum* (23) and for corn resistant to *X. stewartii* (24).

Although *X. campestris* is capable of rapid multiplication in the leaf mesophyll, the abrupt halt of bacterial growth after 48 to 60 hr in both resistant and susceptible genotypes indicated that the bacterium does not enter into a compatible relationship with the leaf parenchyma. The similarity in the leaf responses elicited by *X. campestris* and by various heterologous plant pathogens again suggested a basic incompatibility between *X. campestris* and cabbage leaf parenchyma. A similar incompatibility between leaf tissue of a susceptible host and a virulent bacterium had been described for apple and *Erwinia amylovora* (6). In contrast to the reported independence of the time of symptom appearance from the inoculum dose of incompatible isolates of *Pseudomonas syringae* on bean and cherry leaves (5), the time of symptom appearance in leaf panels of cabbage is inversely proportional to the inoculum dosage of *X. campestris* (Table 1).

Although leaf mesophyll from both resistant and susceptible plants seemed to respond similarly to *X. campestris* and heterologous bacteria, the preferential survival of *X. campestris* near the vein limiting the infiltrated panels (Table 2) indicates a possible basis for the pathogenicity of this bacterium on cabbage. One of the factors determining the pathogenicity of *X. campestris* on cabbage might be its resistance to bactericidal compounds produced during the necrotic reaction at the hydathodes, permitting the pathogen to macerate the xylem vessel terminals and gain entry into the larger xylem vessels.

The infrequent development of systemic black rot lesions after the infiltration of intercostal panels with high numbers of bacteria indicated that the pathogen is not usually able to gain entry into the vascular system from the intercellular spaces of the leaf parenchyma except via the vein terminals in the hydathodes. The failure of *X. campestris* to develop systemically following penetration through the stomates (2, 3) also supports the observation that the hydathodes are the only natural points of entry for the black rot pathogen into the vascular system of cabbage leaves.

The results of this study lead to the conclusion that resistance to black rot in cabbage, conferred by the *f* gene, operates by restricting bacterial multiplication and spread in the xylem and xylem vessel terminals of the hydathodes, and that induced changes in the aromatic metabolism might be responsible for the bacterial restriction.

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