Whole Plant Wound Inoculation for Consistent Reproduction of Black Rot of Crucifers

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ABSTRACT

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Several widely used inoculation techniques were compared for their ability to reproduce black rot of crucifers caused by *Xanthomonas campestris* pv. *campestris*. The comparisons showed that different bacteria not normally pathogenic to crucifers can cause necrosis or maceration of turnip and cauliflower leaves if the bacteria were infiltrated into detached leaves or inoculated into stems of 3-day-old seedlings. When inoculated similarly in leaves and 3-day-old seedlings, *X. c. campestris* also caused symptoms atypical of black rot that were visually indistinguishable from those caused by the other bacteria. In most instances black rot did not develop and was delayed in onset when it did. On the other hand, *X. c. campestris* was able to cause black rot quickly and at virtually all inoculation sites when inoculations were made at the hydathodes or wounds of intact plants. By this technique, *Agrobacterium tumefaciens, Arthrobacter luteus, Clavibacter michiganense* subsp. *michiganense, Erwinia carotovora* subsp. *carotovora, E. herbicola, E. stewartii, E. amylovora, Escherichia coli, Pseudomonas fluorescens, P. putida, P. viridiflava, Serratia marcescens, X. c. zinniae, X. c. begonia, X. c. malvacearum, X. c. oryzae, X. c. transluscens, X. c. vesicatoria*, and *X. c. vitians* failed to cause black rot.

Additional keywords: compromised host defense, pectolytic enzymes.

Xanthomonas campestris pv. *campestris*, which causes black rot, a worldwide disease of crucifers, is specific for members of the Brassicaceae (15,19,21). The bacteria normally invade hosts through wounds or hydathodes (2,16,26). Upon entry, the bacteria spread within the vascular system to which they are confined (3,21,28) and to where they eventually cause the distinct blackening of leaf veins and death and drying of panels of leaf tissue that were delimited by the destroyed veins. Hydathode infections are the major mode of entry in nature, where the bacteria have direct access to the vascular system (2). The only other natural mode of infection is through wounds inflicted by rain, wind, insects, or other agents (17,21).

Stomatal infections have not been observed by *X. c. campestris* to result in black rot, prompting investigators to speculate why the disease cannot be initiated at that site (2,21). Leaf spots of crucifers are not regarded as typical black-rot symptoms and are caused by different pathovars of *X. campestris*, called pathovar *aberrens* (13,24) and pathovar *armoraciae* (12). The lesions that result from these stomatal entries never develop into black rot, and the leaf spots are easily distinguishable from the systemic vein blackening that is typical of black rot. *X. c. campestris*, experimentally introduced by stomatal inoculations, causes soft rots or water-soaking symptoms, especially if the leaves or plants are placed in conditions of high humidity (4). Storage rots or "mushy" rots have been reported in harvested cabbage heads that were preinfected by *X. c. campestris* (17), but these are generally thought to be caused by secondary infections (7). High humidity can facilitate soft rot of nonhost tissues such as potato tuber slices inoculated with *X. c. campestris* (17,21). This pathogen elaborates pectolytic enzymes in culture (6,23), which likely contributes to the soft rot of nonhost tissues in experimental inoculations. A number of *Xanthomonas* strains have been reported that can macerate the excised tissues of various plants (11). In contrast, black rot is a dry rot on whole plants, leaving affected leaves parchment-like or leathery (1,8,17,21), or storage roots hollow and dry (20). Occurrence of soft rot or wet rot in field plants is commonly the result of secondary infections by other microorganisms (21,25,26).

Water-soaking and soft-rot symptoms therefore can interfere and be confused with black-rot symptom analysis. Because atypical symptoms can be produced under experimental conditions, we have tested a number of bacteria on crucifer hosts, utilizing several inoculation techniques reported in the literature to determine an inoculation method suitable for the study of black rot. Our desire was to elicit host symptoms that were natural for black rot and that could be induced only by *X. c. campestris*. Because disease is a process of host and pathogen activities over time, we were interested in an assay that might connect two or more separate phenomena such as bacterial growth and symptom development, or bacterial penetration and subsequent movement in the host. Several variables were considered: reproducibility of results, accurate representation of disease progress, and ease of inoculation of large numbers of plants.

MATERIALS AND METHODS

Media and antibiotics. All bacteria were grown in medium 523 (10) with shaking (221 revolutions per min) at 28 C. When required the medium was supplemented with 1.5% Bacto agar (Difco). Water agar plates were prepared with Bacto agar, the concentrations of which are indicated below (pathogenicity assays). Aqueous stock solutions of rifampicin (Sigma) were filter-sterilized and dispensed into autoclaved media at the desired concentrations.

Host plants. *Brassica campestris* L. 'Just Right' (turnip) seeds were a gift from American Takii Seed Co. *Brassica oleraceae* L. cv. *botrytis* L. 'Early Super Snowball' (cauliflower) seeds were from Park Seed Co. Except where specified differently, all plants were transplanted at 2 wk of age (no true leaves) to separate growth pots (5" diameter). Plants were supplemented with a fertilizer (10-10-10) at the time of transplantation. Plants were grown at 20–30 C in air-conditioned greenhouses, and tests were conducted all year long. Except in seedling tests, all plants were inoculated at the six-leaf stage and only leaves three, four, and five were used in assays.

Bacterial strains. A variety of bacteria were used (Table 1). Rifampicin-resistant bacteria were selected as spontaneous mutants on 523 agar containing 50 μg of rifampicin per ml. Buffer suspensions of the bacteria were prepared after growing the

bacteria overnight in broth and were washed twice with buffer (0.7% NaCl, 1.15% K_2HPO_4, 0.02% KH_2PO_4, 0.02% KCl). After the second wash the cells were resuspended to their original volumes (in buffer), serially diluted, and viable counts were taken.

Pathogenicity assays. Seedling inoculations were essentially as described by Daniels et al (4). Seeds of cultivar Just Right turnip and cultivar Early Super Snowball cauliflower were surface-sterilized by immersion in 1% NaOCl for 30 min, washed several times with sterile, distilled water, and germinated on water agar (1.5%) at 25 C. After 24 hr all of the seeds had germinated. They were transferred to water agar plates (0.7%), supplemented with one-quarter-strength medium 925 salts (10), and placed 1–2 cm apart. The seedlings were inoculated on the third day with the various bacteria. Eight or more seedlings per bacterial strain were used per trial, and six or more trials were conducted. Inoculation was achieved by dipping a sterile nichrome wire into inoculum 5×10^6 colony-forming units (cfu) per ml and then pricking the seedling stem. The seedlings were incubated in sterilized plastic boxes (9 × 22.5 × 32.5 cm; Tri-state Molded Plastics, Dixon, KY) at 25 C and 100% relative humidity.

Infiltration assays of attached and detached leaves were conducted essentially as described by Osbourn et al (14). A plastic syringe (without needle) was used to infiltrate the leaf with bacterial cells by gently pressing the nozzle against the lower surface of a leaf while supporting the leaf on the opposite side. Entire leaf panels were infiltrated.

Hydathode infections were achieved by placing five plants in styrofoam chests with 1–2 cm of standing water in the bottom of each chest. Between 1600 and 1900 hr bacteria (5×10^6 cfu/ml) were misted with a spray bottle over the upward exposed surfaces of the guttated plants until the leaves were wet, and the lids were placed on the chests overnight and removed in the morning. The next evening the lids were replaced again for the night. On the second morning the plants were removed from the chests to greenhouse benches.

Wound inoculations were achieved by dipping a sterile needle into bacterial colonies on agar plates and jabbing the needle into leaf petioles in three spots about 1, 2, and 3 cm below the lamina. Plants were placed back on greenhouse benches for symptom development.

TABLE 1. Bacteria used in the inoculation of cauliflower and turnip plants

Bacterial species	Strain	Host	Source[a]
Agrobacterium tumefaciens	LBA4301 (pTiC58)	Many	DCGG
Arthrobacter luteus	18D1	None	DCGG
Clavibacter michiganense subsp. *michiganense*	493	Tomato	R. Grogan
Erwinia amylovora	1D315	Pear	DCGG
Erwinia carotovora subsp. *carotovora*	3D35	Potato	DCGG
E. herbicola	25D33	None	DCGG
E. stewartii	29D33	Corn	DCGG
Escherichia coli	1222	None	J. Shapiro
Pseudomonas fluorescens	11D49	None	DCGG
P. fluorescens	85-126-2	None	R. Campbell
P. putida	34D4	None	DCGG
P. viridiflava	6D47	Kiwi	K. Cohn
Serratia marcescens	M-61R	None	This paper
Xanthomonas campestris pv. *zinniae*	18D5	Zinnia	M.-T. Lai
X. c. begoniae	19D5	Begonia	W. Wiebe
X. c. campestris	2D518	Crucifers	DCGG
X. c. campestris	2D520	Crucifers	DCGG
X. c. campestris	79-18	Crucifers	R. Campbell
X. c. campestris	81-37	Crucifers	R. Campbell
X. c. campestris	82-3	Crucifers	R. Campbell
X. c. campestris	83-20	Crucifers	R. Campbell
X. c. malvacearum	1D57	Cotton	DCGG
X. c. oryzae	17D51	Rice	S.-T. Liu
X. c. transluscens	10D52	Grains	V. Hall
X. c. transluscens	10D53	Grains	V. Hall
X. c. vesicatoria	6D55	Tomato	DCGG
X. c. vitians	7D53	Lettuce	DCGG

[a] DCGG, Davis Crown Gall Group, University of California, Davis 95616.

RESULTS

Inoculation of seedlings. Most of the 26 bacterial strains that were tested caused no reaction or only a small amount of browning when inoculated into the hypocotyls of turnip seedlings (Table 2). Only *E. carotovora* 3D35, *P. fluorescens* 85-126-2, and all the *X. c. campestris* isolates invaded the seedlings through the wound site and caused the eventual collapse of the plant. In these cases the wound turned dark brown or black in the first few days following inoculation and then discoloration spread up and down the hypocotyl. Within 3–6 days after inoculation the seedlings began to collapse and displayed signs of soft rot or acute water soaking (Fig. 1). Usually, 100% of the plants were killed when inoculated with *E. carotovora* or *X. c. campestris* strains, whereas 60% of the plants were killed by *P. fluorescens*.

Infiltration into detached leaves of cauliflower and turnip. Most bacteria (10 cfu/ml) were unable to cause any effect other than that caused by buffer when they were infiltrated into detached leaves of cauliflower and turnip. On the other hand, several bacteria were definitely able to infect the detached cauliflower leaves. These included *E. amylovora* 1D315, *E. carotovora* 3D35, *P. fluorescens* 85-126-2, *X. c. vesicatoria* 6D55, *X. c. begonia* 19D5, and all the *X. c. campestris* strains. These bacteria were able to cause necrosis of the infiltrated tissue and the subsequent vein discoloration of other areas of the cauliflower leaf (Fig. 2 and Table 3).

A noteworthy heterospecific response of detached leaves is the fact that all of the bacteria that caused vein discoloration were able to cause a wet rot of the infiltrated area and the midveins of inoculated leaves. Often the leaves were rotted to such an extent that they collapsed under their own weight or fell into two parts, tearing at the midvein. In all cases, only those bacteria that were

TABLE 2. Response to bacteria inoculated into the hypocotyl of turnip seedlings

Inoculum	Response[a]
Agrobacterium tumefaciens LBA4301(pTiC58)	B
Arthrobacter luteus 18D1	–
Clavibacter michiganense subsp. *michiganense* 493	B
Erwinia amylovora 1D315	B
Erwinia carotovora subsp. *carotovora* 3D35	+ (100%)
E. herbicola 25D33	–
E. stewartii 29D33	B
Escherichia coli 1222	–
Pseudomonas fluorescens 11D49	–
P. fluorescens 85-126-2	+ (60%)
P. putida 34D4	B
P. viridiflava 6D47	B
Serratia marcescens M-61R	–
Xanthomonas campestris pv. *zinniae* 18D5	B
X. c. begoniae 19D5	B
X. c. campestris 82-3	+ (100%)
X. c. campestris 2D520	+ (92%)
X. c. campestris 79-18	+ (96%)
X. c. campestris 81-37	+ (100%)
X. c. campestris 83-20	+ (100%)
X. c. malvacearum 1D57	B
X. c. oryzae 17D51	B[b]
X. c. transluscens 10D52	B
X. c. transluscens 10D53	–
X. c. vesicatoria 6D55	B
X. c. vitians 7D53	B

[a] Responses on turnip seedlings: a minus sign indicates neither wound irritation nor plant death; a B denotes browning of the wounded tissue with no subsequent damage to the host; and a plus sign indicates the death and soft rot of the seedling. The numbers in parentheses indicate the average total percentage of plants that died. Plants were inoculated in groups of eight per plastic container, and all results are averages of six trials. Plants were observed for 7 days following inoculation. Similar results were obtained for cauliflower seedlings (data not shown).

[b] From University of Hawaii.

infiltrated into leaves were recovered from the water-soaked areas. *P. viridiflava* 6D47 caused leaf death in the infiltrated area only and was not associated with any soft or wet rot or vein discoloration.

Infiltration into attached leaves. Most bacteria (5×10^7 cfu/ml) caused no major response in the cauliflower when infiltrated into leaves in buffer. However, all bacteria, even *E. coli*, were occasionally associated with a mild discoloration (browning) of the infiltrated area. This response did not develop until about 2 wk after inoculation and was usually only visible when the leaves were illuminated from behind. Some bacteria were responsible for localized leaf death in the infiltrated area (Fig. 3 and Table 3). All *X. c. campestris* strains, *P. viridiflava* 6D47, *P. putida* 34D4, and *X. c. malvacearum* 1D57 and *X. c. vitians* 7D53 caused this latter reaction. Only the area of host tissue mechanically infiltrated with bacteria died, and this occurred 3–6 days after inoculation. Necrosis was localized and restricted to the leaf area that became water soaked during the infiltration. Black rot was observed to develop in less than half of the cauliflower plants (Fig. 4) that had been inoculated with *X. c. campestris* by infiltration. This did not happen until about 2 wk after inoculation, with vein blackening and leaf chlorosis spreading out from the infiltrated area. Similar results were obtained for inoculation of turnip and for lower concentrations of bacteria (5×10^6 and 5×10^5 cfu/ml).

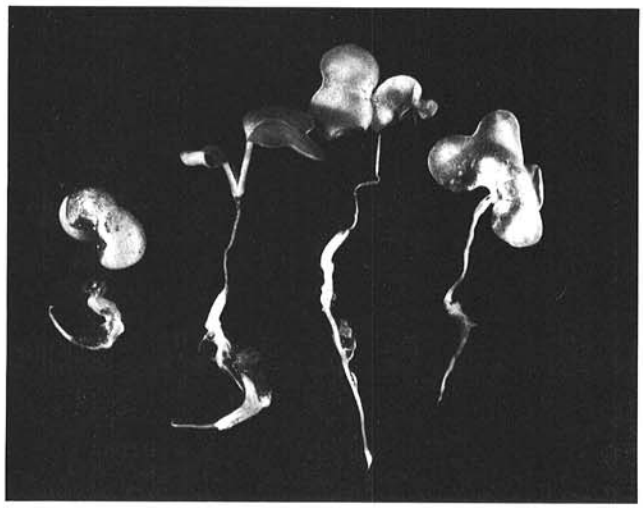

Fig. 1. Hypocotyl inoculation of seedling turnips. The two plants on the left were inoculated with *E. carotovora* 3D35, and the two plants on the right were inoculated with *P. fluorescens* 11D51. Symptom progression is described in the text.

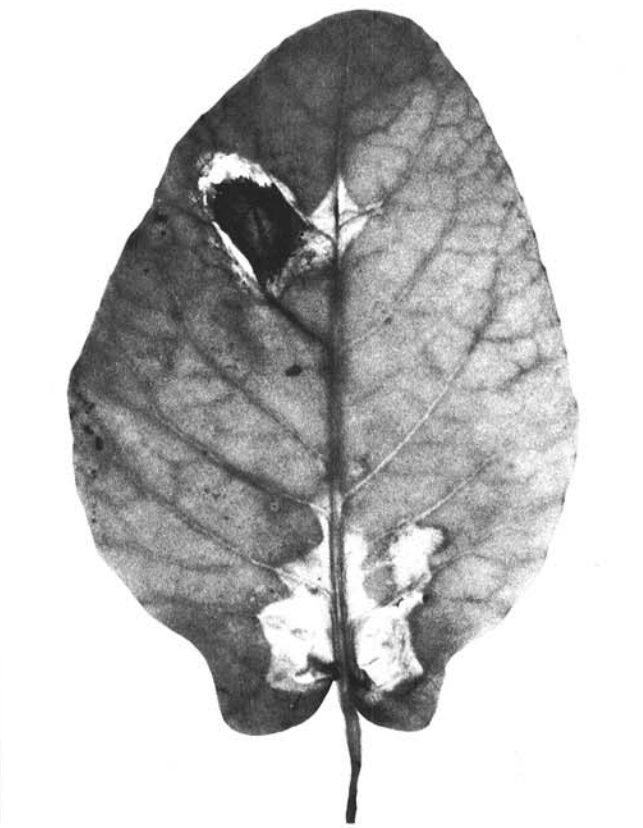

Fig. 2. Soft rot of detached leaves. A cauliflower leaf showing extensive vein discoloration and soft rot caused by *P. fluorescens* 11D51. The dark area of the leaf is where the bacteria were initially infiltrated.

Hydathode inoculation. By using hydathode inoculations, *X. c. campestris* strains were able to cause typical severe black-rot symptoms in cauliflower and turnip. In some cases the vascular tissue of the storage root of turnip plants became severely blackened, but leaf veins were often only mildly discolored. None of the other *Xanthomonas* pathovars or the other bacteria listed in Table 1 caused any visible reaction. Soft rot did not occur.

Wound inoculations. Many of the bacteria seemed to cause slight reactions (e.g., browning) at the wound site. The needle wounds became brown and even split open in a response that was clearly different from that caused by the control inoculations with *E. coli*. Only *X. c. campestris* isolates were able to cause typical black rot on cauliflower and turnip, with the symptoms similar to those that developed in response to hydathode inoculation.

TABLE 3. Comparison of symptoms produced by infiltration of different bacteria into detached and attached leaves

Inoculum	Detached leaf[a]		Attached leaf[a]	
	Cauliflower	Turnip	Cauliflower	Turnip
Agrobacterium tumefaciens LBA4301(pTiC58)	−	−	−	−
Arthrobacter luteus 18D1	−	NT	−	NT
Clavibacter michiganense subsp. *michiganense* 493	−	NT	−	NT
Erwinia amylovora 1D315	+	+	−	−
Erwinia carotovora subsp. *carotovora* 3D35	+	+	−	−
E. herbicola 25D33	−	NT	−	NT
E. stewartii 29D33	−	NT	−	NT
Escherichia coli 85-126-2	−	NT	−	NT
Pseudomonas fluorescens 11D49	−	NT	−	NT
P. fluorescens 85-126-2	+	+	−	−
P. putida 34D4	−	NT	LD	NT
P. viridiflava 6D47	LD	LD	LD	NT
Serratia marcescens M-61R	−	−	−	−
Xanthomonas campestris pv. *zinniae* 18D5	−	−	−	−
X. c. begoniae 19D5	+	+	−	−
X. c. campestris 1D518	+	+	LD, BR	LD, BR
X. c. campestris 1D520	+	+	LD	LD
X. c. campestris 79-18	+	+	LD, BR	LD, BR
X. c. campestris 81-37	+	+	LD	LD, BR
X. c. campestris 83-20	+	+	LD, BR	LD, BR
X. c. malvacearum 1D57	−	−	LD	NT
X. c. oryzae 17D51	−	NT	−	−
X. c. transluscens 10D52	−	NT	−	−
X. c. transluscens 10D53	−	NT	−	NT
X. c. vesicatoria 6D55	+	+	−	NT
X. c. vitians 7D53	−	NT	LD	LD

[a] The responses for both cauliflower and turnip are indicated. A minus sign denotes a response that was no different from leaves infiltrated with buffer or *E. coli*; a plus sign indicates both leaf death at the infiltration site and ensuing soft rot involving leaf veins; LD indicates leaf tissue death localized at the infiltration site; BR means that black-rot symptoms developed at the infiltration site (emanating from the area of leaf death); and NT means that the inoculation was not performed. Plants were observed for 14 days following inoculation.

DISCUSSION

We have demonstrated that black rot is caused specifically by *X. c. campestris* when intact plants (not seedlings) are used. Our studies show two important observations: atypical symptoms such as soft rot and leaf death develop by infiltration of leaf panels; and *X. c. campestris* and other bacteria can cause soft rots of seedlings and excised plant parts, and not black-rot symptoms.

In our study, *X. c. campestris* was severely reduced in its ability to cause black rot when mechanically infiltrated into leaves on plants, and instead caused localized tissue death that occasionally gave rise to black rot. Therefore, because black rot is elicited by this means only in a minority of cases and because bacteria other than *X. c. campestris* cause similar tissue death, we conclude that this inoculation technique is not suitable for black-rot studies. Use of such techniques runs the risk of misinterpreting the pathogenic phenotype of *X. c. campestris*.

In natural infections, *X. c. campestris* cells are largely confined to the xylem and rarely contact parenchymatous cells (3,5,28). These bacterial cells that break out of the xylem are almost exclusively confined to the vascular bundle and are seldom, if ever, found in the mesophyll or palisade tissues of the leaf (21). Electron microscopic examinations have never detected these bacteria outside of xylem vessel elements (27). Pockets of bacteria can form in the pith of stems, but this occurs only in advanced cases of the disease after the integrity of the plant has been severely compromised. Intrusions into the leaf mesophyll do occur, but are rare and are quickly curtailed because the bacteria cannot grow into the leaf lamina (17,21).

Assessment of pathogenicity by using detached leaves can no longer be considered a counterpart to natural infection because several bacteria (Table 3) were able to cause vein discoloration such as black rot in excised leaves when inoculated by pressure infiltration. The easiest explanation for this is to assume that the bacteria were able to grow within the vascular system of the detached leaves and that vein discoloration resulted when the bacteria degraded the vascular tissues. This discoloration was associated with the soft rot of the leaf and was not observed in leaves attached to the plant when the bacteria were inoculated similarly. Similar observations have been made with *Erwinia chrysanthemi* PEL, LPS, and siderophore mutants in detached leaves of African violets (D. Expert, pers. commun.)

In seedling inoculations, the three different bacteria used in our studies (including *X. c. campestris* and a saprophytic strain of *P. fluorescens*) were able to kill turnip seedlings in the absence of any black-rot symptoms (Table 2). Such inoculations caused water soaking and collapse of the seedlings. Crucifer seedlings have been studied in relation to black rot, and vein blackening has been reported (9,18,22). However, the conditions of our assay were such that relative humidities approached 100% and sterilized seeds were used. These two conditions, taken together, may create an especially benign condition for microbial growth on plant surfaces. Thus, undeveloped seedling defenses coupled with exotic growth conditions may allow ordinarily harmless bacteria (as well as *X. c. campestris*) to proliferate in such plants and cause soft rot. Similar rotting, caused by *X. c. campestris*, has been reported by other workers when similar experimental conditions were employed (22). As in he cases above, we conclude that this assay is not suitable for our purposes because it does not distinguish *X. c. campestris* from all other bacteria and it did not result in typical black-rot symptoms.

In contrast to infiltration and seedling inoculations, hydathode and wound inoculations were successful in causing black rot on cauliflower. Occasionally a plant did not develop symptoms using this latter procedure, but this was unusual. The wound inoculation method was considerably easier to perform than the hydathode

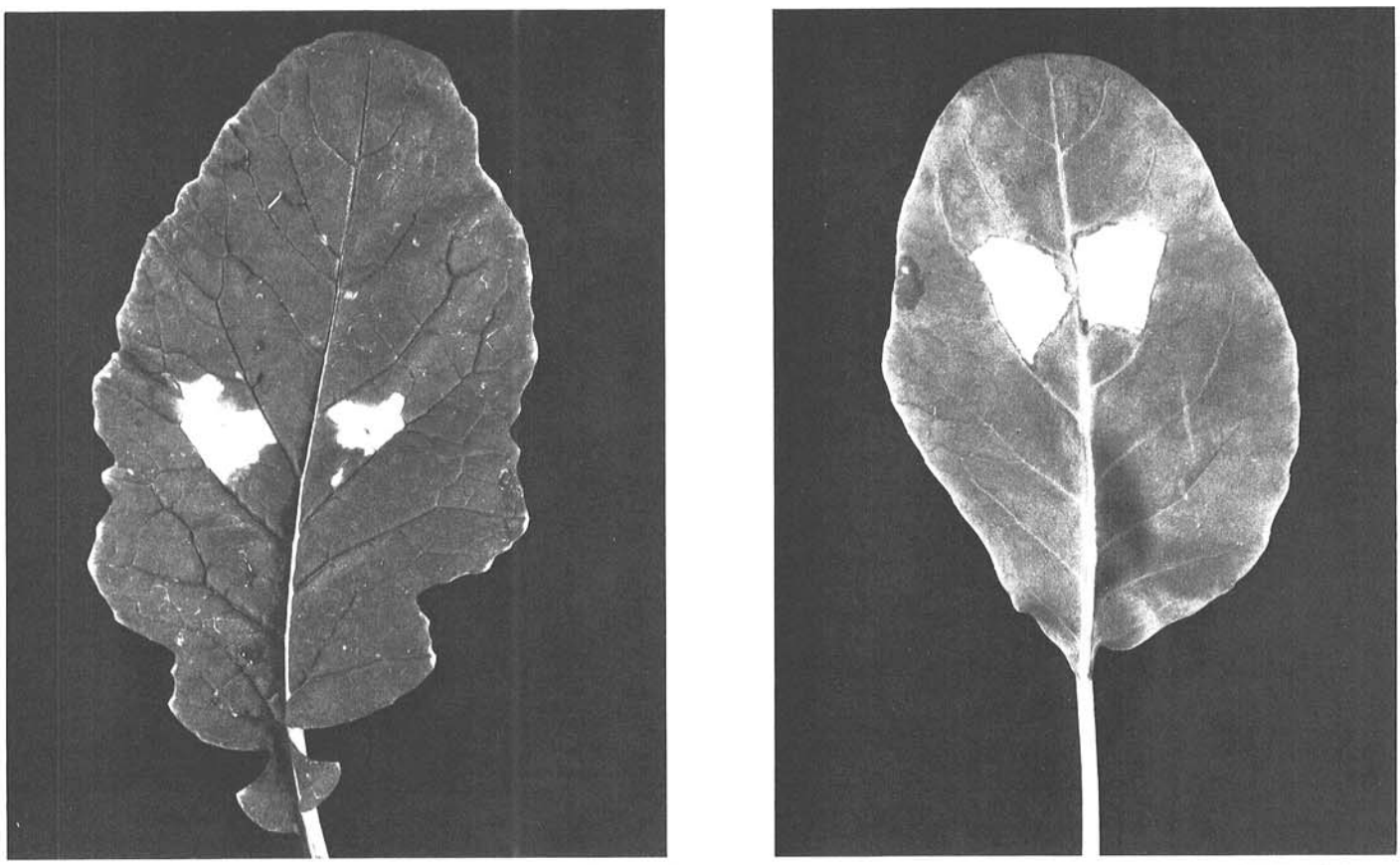

Fig. 3. Localized leaf death of attached leaves. Turnip (**A**) and cauliflower (**B**) leaves infiltrated with *Xanthomonas campestris* pv. *campestris* 2D531, 10 days after inoculation. The irregular white areas of the leaf are where the bacteria were infiltrated. Black-rot symptoms were not present here and generally did not develop from this inoculation procedure (see Fig. 4).

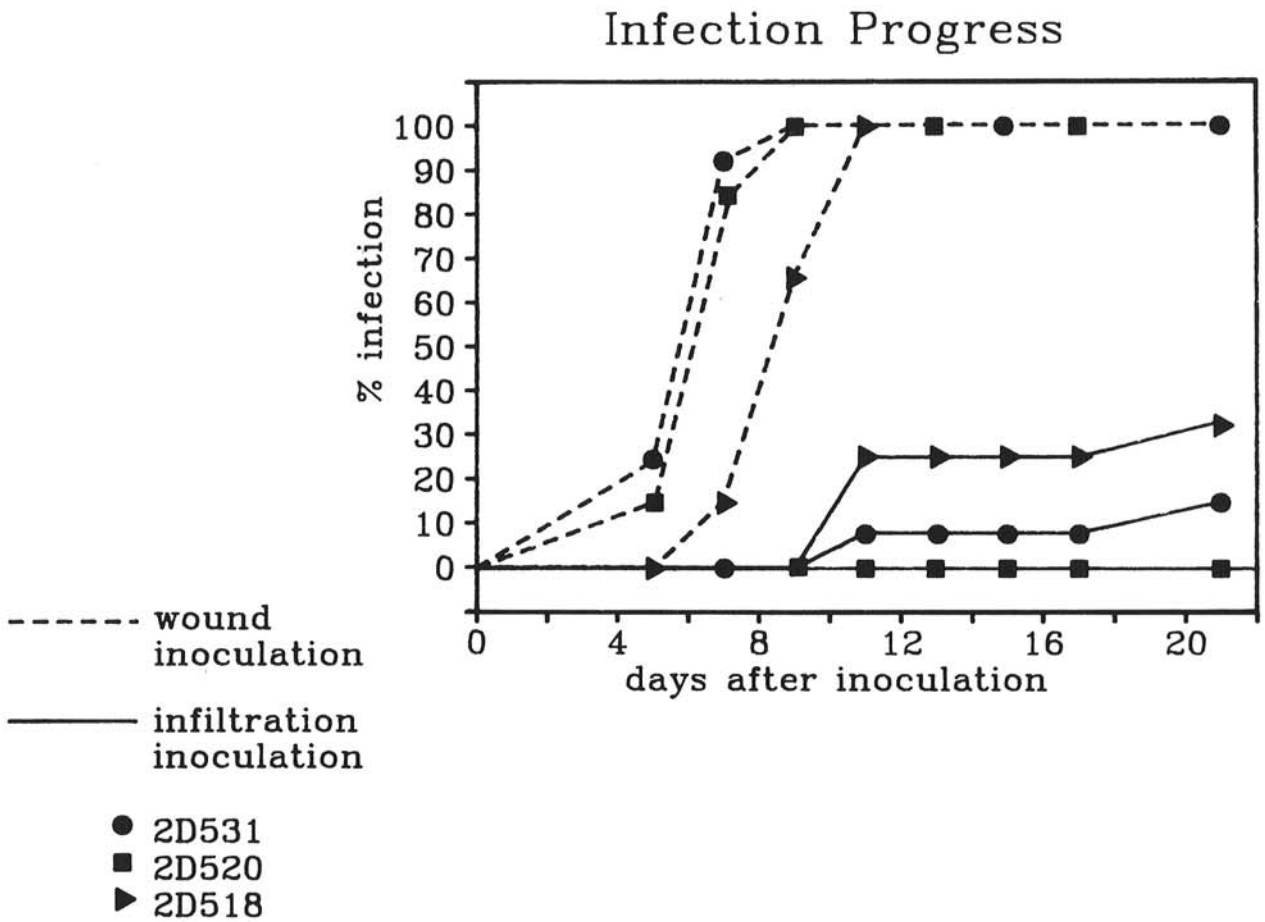

Fig. 4. Infection progress of wound inoculations and leaf infiltration inoculations in cauliflower. Wound inoculations quickly result in black rot at virtually all inoculation sites (6–10 days), whereas infiltration inoculation does so in the minority of cases. In both procedures, the leaves usually abscise by the end of the third wk after inoculation. *X. c. campestris* 2D520 was never observed to cause black rot when infiltrated into the mesophyll (33 inoculations), whereas black rot always resulted when the same strain was inoculated into wounds.

method and required less attention to the plants. Also, wound inoculation provided the advantage of predictability: when symptoms developed they always were on the inoculated leaf, whereas with the hydathode inoculation it was unclear which leaves would develop symptoms. Both of these methods shared the advantages that only black-rot symptoms developed and no other bacteria could incite a similar effect.

Special care was taken to cause the plants to form guttation droplets in the hydathode inoculation; i.e., enclosure overnight in a humid environment. This step probably does not represent field conditions where, although guttation drops frequently develop, humidity is seldom constant at 100% and the soil is seldom flooded. This mode of inoculation is similar to that reported by other workers (2,9), and no unusual symptoms were observed. Importantly, bacteria normally nonpathogenic to crucifers were not able to infect the plants, unlike the results in the infiltration and seedling assays.

Based on these observations, we propose that the intact plants such as cauliflower and turnip possess natural defenses against nonspecific bacteria that intrude their tissues. Detached parts of plants such as leaves may no longer retain or invoke these defenses. This may also hold true for young seedlings, which may have underdeveloped defenses. Thus, with their natural defenses compromised, young seedlings and detached leaves should be used judiciously in the assessment of virulence and pathogenicity.

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