Factors Influencing Black Rot Lesion Development in Resistant and Susceptible Cabbage

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ABSTRACT

The effects of light, temperature, and inoculum level on lesion development on resistant and susceptible cabbage were determined. Resistance in veins was distinguished from that in hydathodes by inoculating injured vein endings and noninjured hydathodes. The hydathode was implicated as the site at which resistance normally operates. In resistant plants, the inoculation of injured vein endings resulted in black rot lesions, but the cessation of lesion enlargement 9 to 12 days after inoculation indicated an induced inhibition of bacterial multiplication in the major veins. Multiplication of *Xanthomonas campestris* in the veins of resistant and susceptible plants paralleled lesion progress. In resistant plants, inhibition of both lesion progress and bacterial growth were more pronounced at 20 than at 28 C. The generation times of *X. campestris* were the same in xylem fluid from resistant and susceptible genotypes. Both genotypes had similar percentage infection at varying inoculum doses when either veins or hydathodes were inoculated, suggesting that there was no preformed resistance in the cabbage. The concomitant appearance of lesions in both resistant and susceptible plants after vein inoculation together with the subsequent divergence in the rate of lesion growth also suggested that a period of time was required after inoculation for resistance to become operative. The decline of resistance under low light intensity (600 ft-c) and the delay of induced resistance at temperatures above the optimum for cabbage growth (22 to 24 C) indicated the importance of the physiologic condition of the host in resistance.

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The causal organism of black rot, *Xanthomonas campestris*, normally enters the cabbage leaf through the hydathodes, and under favorable conditions spreads systemically in the xylem vessels throughout the susceptible plant. Vessels in which the bacteria multiply turn black. In leaves the intercostal regions become chlorotic, and these areas desiccate after they become surrounded by blackened veins (12). In stems, petioles, and heads, vascular blackening can occur without external symptoms (2, 11).

In field-grown cabbages containing the major *f*-gene for black rot resistance derived from the cultivar Early Fuji, the symptoms are either a localized marginal necrotic lesion of variable size, with a distinct dark rim, or a minute black area at the infected hydathode (14). These same reactions are often the first visible symptoms on many susceptible cultivars after inoculation in the field, but within susceptible plants, the bacteria usually spread beyond the dark margins of the necrotic lesions and form characteristic chlorotic, v-shaped black rot lesions (14). In both genotypes, the larger veins delimiting the necrotic lesions were usually free of the blackening characteristic of veins in lesions. The rapid collapse of interveinal tissues suggested an extensive multiplication of the bacteria in the leaf parenchyma similar to that observed by Smith (10) and Meier (8) in the hydathode regions prior to invasion of the vein terminals.

During black rot epidemics induced artificially in the field for the purpose of genetic screening, it was repeatedly observed that susceptible type lesions developed on resistant plants after injury by insects or hail (14). This observation indicated that the primary locus of resistance may be located in the hydathode region, even though *X. campestris* appeared capable of multiplying within the vessels of resistant plants.

This study was undertaken to examine the development of black rot lesions on genetically defined resistant and susceptible cabbage lines to evaluate the role of the hydathode in resistance. Since temperature (2), light (3, 7, 9), and inoculum dose (1, 15) are important factors in the development of black rot and other bacterial plant diseases, the influence of these factors on black rot lesion development was compared on resistant and susceptible plants.

MATERIALS AND METHODS.—Plants of a black rot-resistant cabbage inbred line, *Brassica oleracea* L. var. *capitata* L., with a black rot rating (BR) of 1 (14) and homozygous for the *f*-gene for resistance derived from Early Fuji, and plants of the susceptible F_1 hybrid cultivar Sanibel (BR of 4) were seeded in Ottawa silica sand and transplanted after 2 weeks into 4-inch pots containing a 1:2 peat-soil mixture. In three experiments, black rot-tolerant F_1 plants with intermediate susceptibility from a Golden Acre X Early Fuji cross were also included.

Plants were grown for 7 weeks in a growth room at 16 and 24 C alternating with a 12-hr dark/light photoperiod under 1,700 ft-c cool-white fluorescent light mixed with incandescent bulbs (1 w of incandescence for every 6 w of fluorescence). Three to four days prior to inoculation, plants were transferred to constant temperature rooms in which the subsequent experiments were to be conducted.

Plants used in light intensity experiments were grown for 12 weeks at 24 C before spray inoculation.

Throughout this study, strain B 87-S 2 of *X. campestris* was used (12). The pathogen was maintained on potato-dextrose agar, and cultures for inoculum were grown for 24 hr in shake culture in 125-ml Erlenmeyer flasks containing 30 ml of Husain & Kelman's medium (4). Bacteria were centrifuged for 10 min at 1,085 *g*, then resuspended in sterile distilled water. The number of bacterial cells was adjusted to an A_{620} of 0.5 (ca. 10^9 cells/ml). Other cell concentrations were calculated by dilution factors from this density. The accuracy of this method was checked each time by viable cell counts.

To compare the expression of resistance and the symptom development on cabbages in the growth room with that observed in the field (14), 20 plants of each line were induced to guttate in dew chambers and inoculated by atomizing a bacterial suspension (A_{620} of 0.5) over the guttating leaves, simulating field inoculations (14). The plants were dried slowly, permitting the bacterial cells in guttation droplets to enter the hydathodes. After inoculation, eight resistant, tolerant, and susceptible plants were kept at 500 to 700 ft-c to determine the effect of low light intensity on the expression of resistance and on lesion development. Lesion development was observed for 16 days when lesion types were recorded.

To evaluate the effect of temperature on lesion development after introduction of the bacteria directly into the xylem, four resistant and four susceptible plants were inoculated at the vein endings as described by Sutton & Williams (12) and placed at 16, 20, 24, and 28 C. Four fully expanded leaves on each plant were inoculated at the endings of the midvein and four lateral veins, two on each side of the leaf. After 9, 12, 15, and 18 days, the advance of vein blockage was determined by measuring the point of eosin blockage from the point of inoculation. For this measurement, petioles of excised leaves were immersed in a 1% aqueous solution of Eosin Y for 1 hr. Four leaves, one from each plant, but from the four different leaf positions, were sampled on each date for each temperature and host line.

Multiplication of *X. campestris* in the veins of resistant and susceptible plants was determined at 20 and 28 C after vein inoculation with 10^9 cells/ml. Bacteria were periodically sampled for dilution plating by punching out the first 3.5 mm of the inoculated veins, using a No. 1 cork borer. At each sampling time, six leaf discs from six plants of each line were crushed separately in 2 ml of sterile distilled water, using a Van Tenbroek homogenizer. The numbers of viable bacteria in each leaf disc were determined by dilution plating in a tetrazolium chloride medium (5).

In vitro growth rates of *X. campestris* were determined in filter-sterilized xylem fluid of healthy resistant and susceptible plants and, as a control, in Husain & Kelman's medium (4). We collected xylem fluid in beakers by permitting the sap to exude from cut stem ends of cabbage grown in pots. Bacterial growth was determined in 50-ml Erlenmeyer flasks containing 10 ml of the various media incubated on a reciprocating shaker at 29 C. For viable cell counts, 0.1-ml aliquots were removed from the flasks at 5- to 8-hr intervals over a period of 36 hr for dilution plating.

To determine the effect of temperature on lesion development after the introduction of the bacteria through the hydathodes, eight resistant and eight susceptible plants were inoculated by placing 1 μliter of bacterial suspension (ca. 10^6 cells) on guttating hydathodes. Guttation was induced by putting the plants in dew chambers for 12 hr at 20 C. The hydathodes at the endings of the midrib and six lateral veins were inoculated on six leaves/plant. The plants were then dried for 2 hr before they were returned to the respective growth rooms at 16, 20, 24, and 28 C. Lesion development and eosin blockage was measured at 10, 14, and 18 days after inoculation.

To evaluate the effects of inoculum concentration on the progression of lesion formation, resistant,

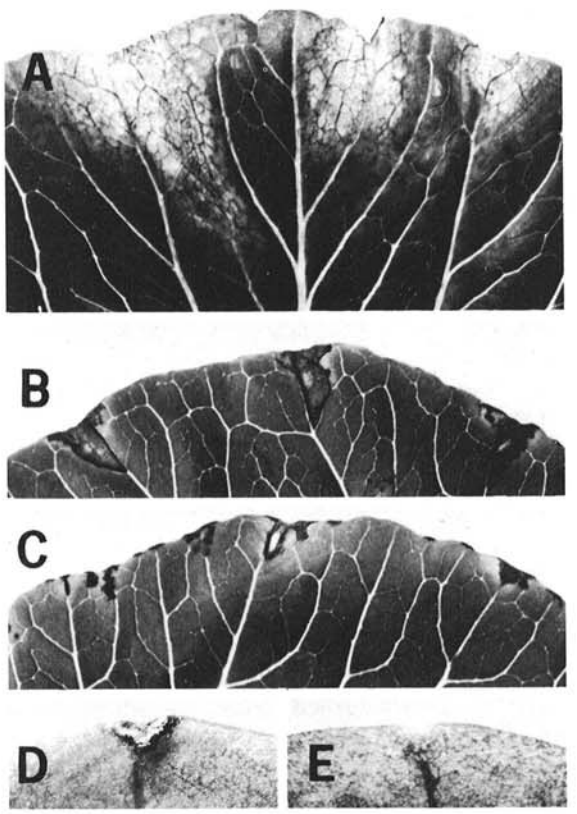

Fig. 1. Black rot lesion types after inoculation of black rot-resistant cabbage with *Xanthomonas campestris* either at injured vein endings (A) or at hydathodes (B, C, D). **A)** Susceptible type lesion showing characteristic v-shape, vein blackening, and diffuse margin. **B, C, D)** Restricted marginal lesions showing: **B)** marginal necrotic intercostal regions with dark border; **C)** marginal necrotic lesions of variable size; **D)** necrotic hydathode region (× 14). **E)** Normal hydathode region (× 14).

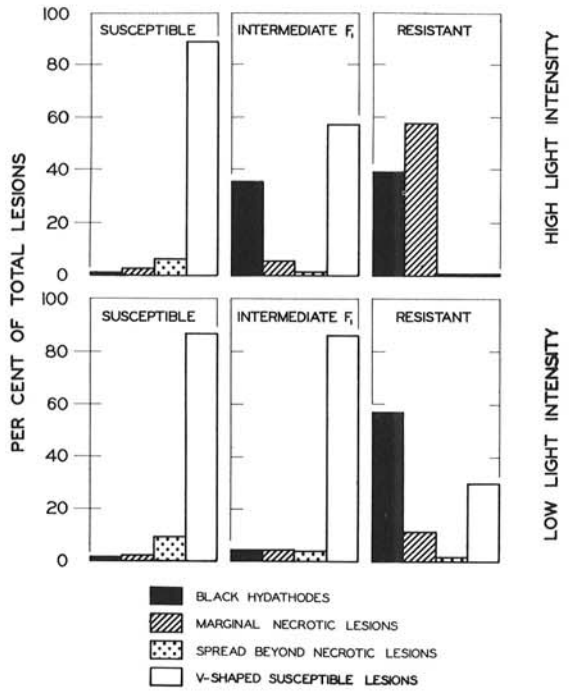

Fig. 2. The influence of light intensity on black rot lesion types in resistant, intermediate, and susceptible cabbages. Lesion types were recorded 16 days after inoculation with *Xanthomonas campestris* through the hydathodes at 24 C. High light intensity = 1,700 ft-c, low light intensity = 500-700 ft-c.

intermediate, and susceptible plants were inoculated at hydathodes and vein tips at 24 C with $10^{5.8}$, $10^{7.3}$, $10^{8.8}$, and $10^{9.9}$ cells/ml. Sixteen plants of each line were divided into four equal groups. Four leaves of each plant were inoculated. The four concentrations of inoculum were distributed over the 16 leaves within each group of plants according to a latin square design. Each leaf was inoculated at the hydathode of the midrib and of three lateral veins on one side of the leaf. The three corresponding vein endings on the other side of the leaf were vein-inoculated after the hydathode droplets were drawn back into the leaf. The progression of tissue collapse, necrosis, and vein darkening were recorded 4, 7, 12, and 17 days after inoculation. At the same time, the per cent of successful infections was determined.

RESULTS.—The lesion types developing on resistant, intermediate, and susceptible plants after spray inoculation of guttating hydathodes in the growth room at 24 C under 1,700 ft-c (high light) were comparable to those observed in the field. Under 1,700 ft-c, only restricted necrotic lesions formed on resistant plants, and the systemic invasion of the host by the pathogen was prevented (Fig. 1-B, C, D, E, 2). Under 500 to 700 ft-c (low light), the pathogen was not restricted in its spread on resistant plants, and over 30% of the lesions were of the spreading type with a chlorotic margin (Fig. 1-A, 2).

On tolerant plants with intermediate susceptibility, the proportion of spreading lesions was greater under low light intensity than at high light intensity, whereas on susceptible plants at both light intensities, more than 95% of the lesions were of the spreading type (Fig. 2). The proportion of necrotic lesions on susceptible plants was higher at 6 to 8 days after inoculation than at 16 days, but the bacteria usually were able to break out of the necrotic areas to form v-shaped spreading lesions by 16 days after inoculation, leaving less than 5% restricted lesions (Fig. 2).

The average numbers of lesions per resistant, intermediate, and susceptible plant were, respectively, 52, 23, and 60 under high and 41, 26, and 68 under low light intensity. Although the numbers of lesions within both homozygous-resistant and susceptible genotypes are similar (Table 1), the consistently lower per cent of infections in the intermediate plants is unexplained.

After vein inoculation, lesion development was recorded as progression of eosin blockage in the inoculated veins (Fig. 3), whereas after hydathode inoculation, symptom development was recorded as progression of vein blackening or tissue collapse and necrosis, since eosin blockage was usually absent around the marginal necrotic lesions (Fig. 1-B).

Inoculation of injured vein endings on resistant plants resulted in the formation of v-shaped chlorotic lesions (Fig. 1-A) at 16, 20, 24, and 28 C, whereas inoculation of hydathodes resulted in only restricted necrotic lesions (Fig. 1-B, C, D) at all temperatures. On susceptible plants, v-shaped lesions formed at all temperatures following both inoculation methods.

The rate of lesion development after vein inoculation was slower on resistant than on susceptible plants (Fig. 3). However, the relative

TABLE 1. Per cent infection following vein and hydathode inoculation of resistant, tolerant, and susceptible cabbage plants with different inoculum levels of *Xanthomonas campestris*

Viable cells/ml of inoculum Log base 10	% Infection[a]		
	Cabbage genotypes		
	Resistant	Tolerant	Susceptible
	Vein inoculation		
5.8	19[b]	27	15
7.3	58	83	72
8.8	100	100	100
9.9	100	100	100
	Hydathode inoculation		
5.8	14[c]	6	3
7.3	73	55	70
8.8	72	37	58
9.9	66	44	80

[a] Both restricted necrotic and spreading chlorotic lesions were counted as successful infections.
[b] Based on 48 inoculations.
[c] Based on 64 inoculations.

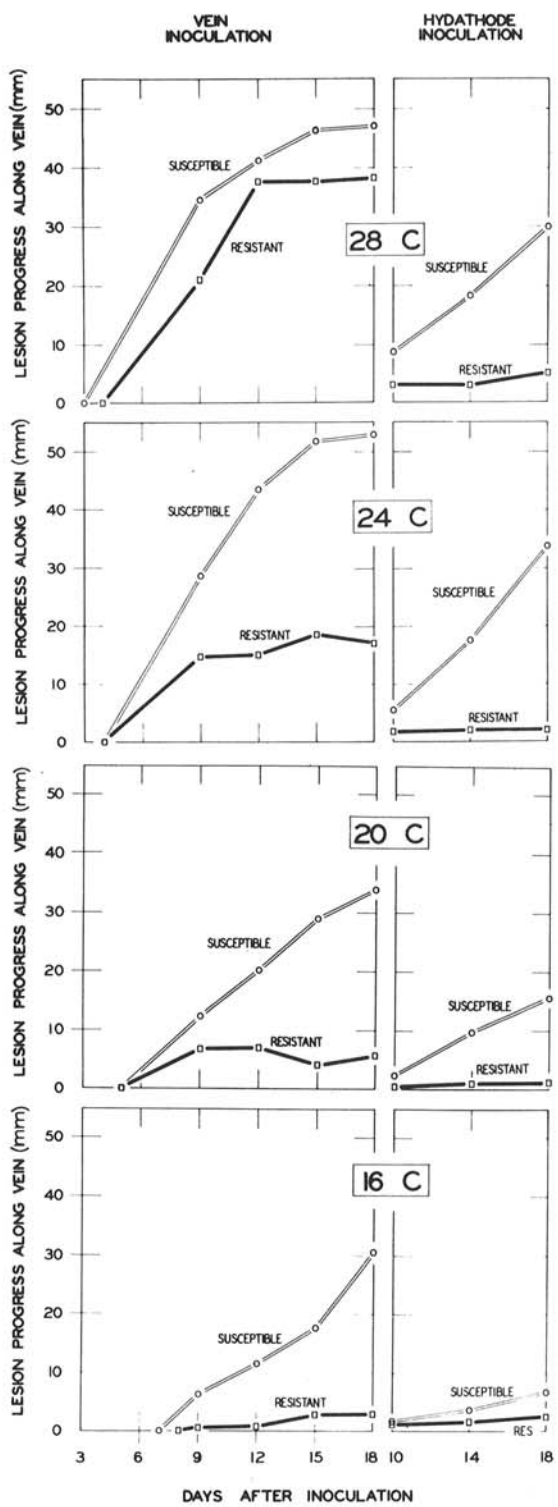

Fig. 3. The development of black rot lesions after inoculation of veins and hydathodes of cabbage leaves with *Xanthomonas campestris* at different temperatures. Extent of vein blockage to eosin movement was measured for vein inoculation; extent vein blackening or tissue collapse and necrosis was measured for hydathode inoculation.

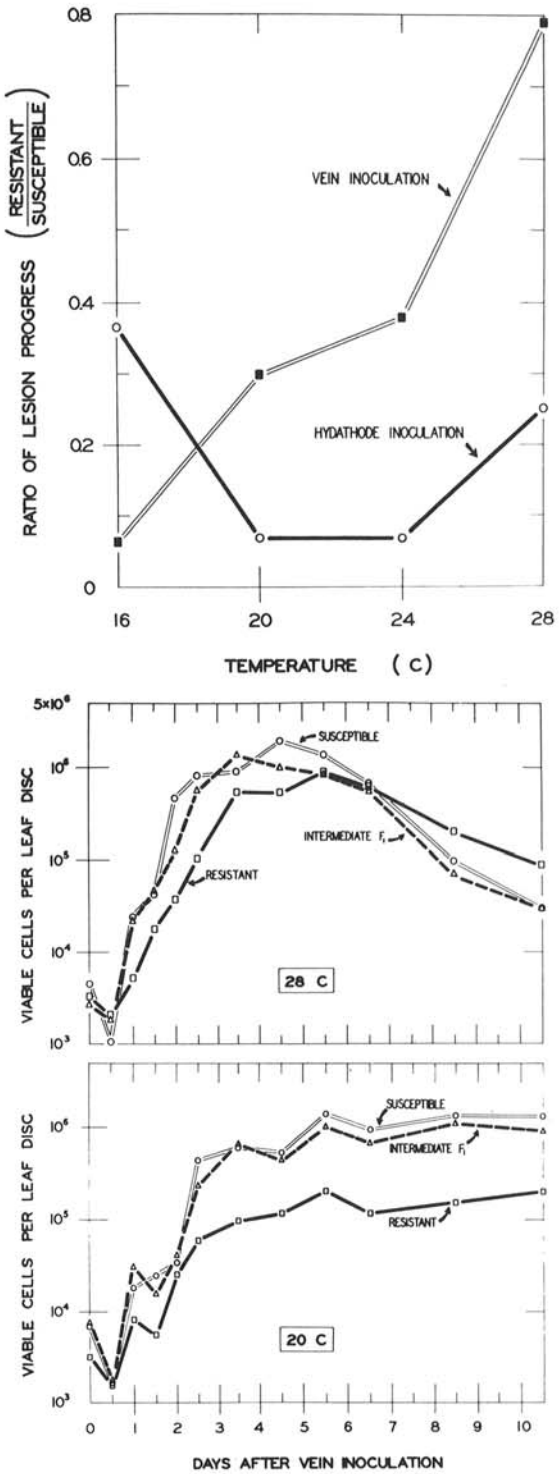

Fig. 4. (Above) The effect of temperature on the ratios between lesion growth on resistant plants and that on susceptible plants following vein and hydathode inoculation of cabbage leaves with *Xanthomonas campestris.* (Below) Viable cell counts of *X. campestris* at periodic intervals in vein endings of resistant, intermediate, and susceptible cabbage leaves at 20 and 28 C after inoculation with 10^9 cells/ml.

advance of the lesions on resistant plants compared to that on susceptible plants increased greatly with increasing temperature. To obtain a numerical expression for the relative rate of lesion progress at each temperature, the measurements made on the four dates on resistant plants were combined and divided by the combined measurements on susceptible plants (Fig. 4, above). The ratios were 0.06, 0.30, 0.38, and 0.79 for 16, 20, 24, and 28 C, respectively. These ratios were slightly higher (0.10, 0.36, 0.45, and 0.88) when they were based on the percentage of each inoculated vein which was blocked, since the leaves of resistant plants were somewhat smaller than those of susceptible plants.

The ratios obtained for hydathode inoculation (Fig. 4, above) reflected the size of the marginal necrotic lesions on resistant plants relative to the size of the v-shaped lesions on susceptible plants. The ratio was lowest (0.07) for 20 and 24 C. The value of 0.25 at 28 C indicated that relatively large marginal necrotic lesions were formed on resistant plants at this temperature, whereas the ratio of 0.37 at 16 C was largely due to the small susceptible lesions formed on susceptible plants at this temperature (Fig. 3). The shape and size of the marginal necrotic lesions appeared to correspond to the shape and size of the water-soaked areas which could be observed at night on healthy plants extending from the leaf margin along the vein for some distance.

After vein inoculation at 20 and 28 C, bacterial growth was faster and reached higher numbers in susceptible than in resistant plants, whereas bacterial growth in tolerant plants was nearly identical to that in susceptible plants (Fig. 4, below). The differences in bacterial growth were more pronounced at 20 C, where the maximum populations reached were 7 times higher in susceptible than in resistant plants. At 28 C, the populations in susceptible plants were only 2.5 times higher than those in resistant plants. At both temperatures, the differences in bacterial populations between resistant and susceptible plants were statistically significant at the 1 to 5% level: for the 1- to 4.5-day samples at 28 C, and for the 1.5-day and the 2.5- to 10.5-day samples at 20 C.

The mean generation times of *X. campestris* in the logarithmic growth phase were 59 and 54 min, respectively, in buffered (0.02 M phosphate buffer, pH 7) and unbuffered (pH 6.3) xylem fluid of susceptible plants and 50 min in both buffered and unbuffered (pH 6.5) xylem fluid of resistant plants, compared with 54 min in Husain & Kelman's medium. The lag phases were 6 hr in the artificial medium and 11 to 13 hr in the various natural media.

When resistant, intermediate, and susceptible plants were vein-inoculated with $10^{5.8}$, $10^{7.3}$, $10^{8.8}$, and $10^{9.9}$ cells/ml, the lesion measurements at 4 days (Fig. 5) were more dependent on the inoculum dose than on the genotype of the host line. The subsequent rate of lesion spread was virtually independent of the inoculum dose and the same for susceptible and intermediate plants, whereas the rate of lesion growth was significantly slower on resistant plants. For both resistant and susceptible lines, the inoculum density of $10^{8.8}$ cells/ml gave rates of lesion growth and times of symptom appearance approximately midway between those produced by $10^{7.3}$ and $10^{9.9}$ cells/ml. The concentration of $10^{5.8}$ cells/ml produced too few lesions for meaningful averages (Table 1).

The appearance of lesions after hydathode inoculation with the four inoculum levels was inconsistent at any level and highly variable among the bacterial concentrations, but the size of the developing lesions was consistent with respect to the host genotypes. Hence, data for the three higher inoculum levels was pooled in Fig. 5. In contrast to the results from vein inoculation, lesion progress in hydathode-inoculated F_1 plants was intermediate between susceptible and resistant plants, rather than similar to susceptible plants.

To determine the effect of inoculum density on the percentage of infections on resistant, intermediate, and susceptible plants, the per cent infection was recorded 17 days after vein and hydathode inoculation with $10^{5.8}$, $10^{7.3}$, $10^{8.8}$, and $10^{9.9}$ cells/ml (Table 1). With vein inoculation 100% infection could be obtained with $10^{8.8}$ and $10^{9.9}$ cells/ml on all three lines, whereas with hydathode inoculation methods, the per cent infection at different inoculum levels was not correlated with resistance.

DISCUSSION.—Within cabbage lines containing the *f*-gene for black rot resistance, the hydathode has been implicated as the site where resistance normally and most effectively operates. Only when bacteria are introduced via the hydathodes is resistance fully and rapidly expressed.

The observation that both the homozygous-resistant and -susceptible genotypes produced the same percentages of infections at varying inoculum doses (Table 1) at vein endings or the hydathodes indicated the absence of preformed barriers to the entrance and the initial establishment of the bacterium. Similarly, the equal growth rates of *X. campestris* in the xylem fluids from resistant and susceptible genotypes seem to rule out the presence of preformed antibacterial compounds in the xylem vessels of noninfected plants. The consistently lower percentage of infections in heterozygous intermediate plants (Table 1) is unexplained.

Although *X. campestris* initially multiplies actively within the veins of resistant plants, the eventual inhibition of both lesion growth and bacterial multiplication in the host strongly indicates that a resistance mechanism is induced in the host by the bacterium.

Within veins of resistant plants, bacterial growth and the induction of resistance are opposing factors in which temperature is the most critical variable for lesion development. Temperatures most suitable for cabbage growth (20 to 24 C) are below the optimum for bacterial growth, and favor the rapid induction of resistance. On the other hand, temperatures which promote optimum bacterial growth (30 to 32 C) (11) are well above the optimum for cabbage growth, and apparently delay the expression of resistance. Hence,

the inhibition of both lesion formation (Fig. 3, 4, above) and bacterial multiplication (Fig. 4, below) is more pronounced at 20 than at 28 C.

In resistant plants, the decrease of inhibition of both lesion growth and bacterial multiplication with increasing temperatures (Fig. 4) is similar to the breakdown of resistance in cabbage to *Fusarium oxysporum* f. sp. *conglutinans* (13). The polygenic

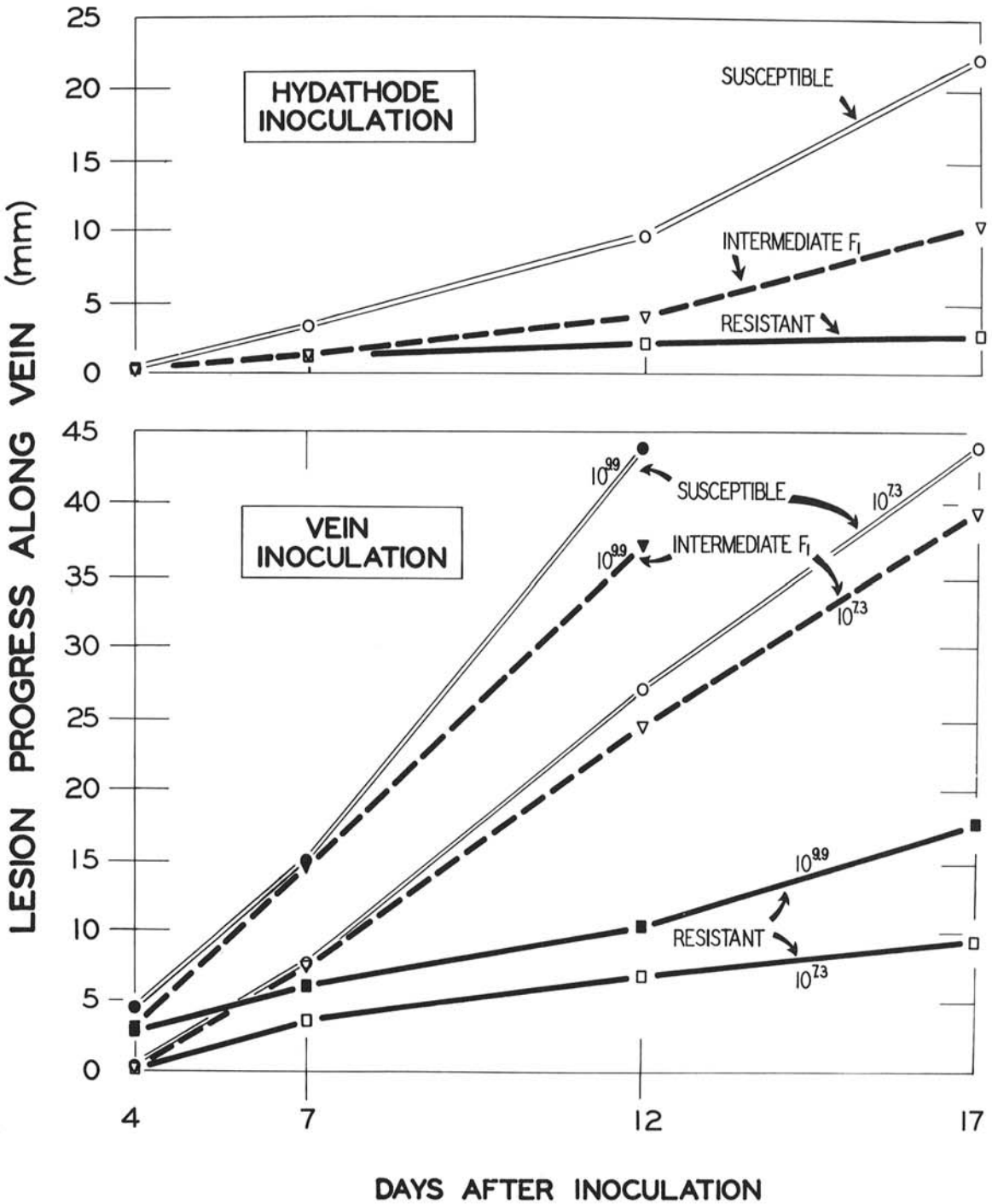

Fig. 5. The development of black rot lesions on resistant, intermediate, and susceptible cabbage leaves at 24 C following vein inoculation with $10^{7.3}$ and $10^{9.9}$ cells/ml of *Xanthomonas campestris* and after hydathode inoculation. For hydathode inoculation, readings for $10^{7.3}$, $10^{8.8}$, and $10^{9.9}$ cells/ml were pooled.

type-B resistance to *Fusarium* breaks down above 22 C, and the monogenic type-A resistance, above 26 C. Although the effects of temperature are similar for black rot resistance after vein inoculation and for yellows resistance, the mechanisms of resistance are likely to differ, since the black rot-resistant genotypes used in this study were susceptible to yellows.

The breakdown of black rot resistance under low light (Fig. 2), as well as the delay of induced resistance at 28 C (Fig. 3), point to the importance of the physiological condition of the host in resistance. Decrease of resistance under low light has also been reported for resistance in potato (9) and tomato (7) to *Pseudomonas solanacearum*, although the same bacterium was reported to cause faster wilting of susceptible potato plants under high light intensity (3). Light has also been shown to be a critical factor in the induction of the hypersensitive reaction in tobacco leaves to race 2 of *P. solanacearum* (6).

The concomitant appearance of lesions on both resistant and susceptible plants after vein inoculation together with the subsequent divergence in the rates of lesion growth (Fig. 3, 5) again suggest the absence of preformed resistance to *X. campestris*, and indicate that a period of time is required after inoculation for resistance to become operative. The delay of lesion appearance at low inoculum levels (Fig. 5) and at low temperatures (Fig. 3) is undoubtedly due to the longer time required for the bacterial populations to reach the levels required to initiate symptom expression. The significant differences in bacterial numbers 24 hr after vein inoculation of resistant and susceptible plants at 20 and 28 C (Fig. 4, below) imply that the induction of resistance begins within 24 hr of inoculation.

Although it is not known whether the temperature-independent resistance at the hydathode and the temperature-dependent induced resistance in the veins are the same, it is possible that they are similar. Bacteria introduced directly into the xylem are in a very different environment than those which enter the intercellular spaces of the hydathode parenchyma. Perhaps by comparing in more detail the responses of the tissues in the hydathode region with those in the veins to the presence of *X. campestris*, a better understanding of the respective roles of the hydathode and the vein in black rot resistance can be gained.

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