Resistance of *Phaseolus* Line WBR 133 to *Pseudomonas syringae*

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ABSTRACT

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Phaseolus line WBR 133 has a high degree of resistance to *Pseudomonas syringae*, causal agent of bacterial brown spot of bean. Histologic leaf imprint, diffusive resistance porometric, and scanning electron microscopic studies showed no differences between WBR 133 and Tenderwhite, a susceptible cultivar, that would affect penetration of the bacteria into the leaf. In vivo multiplication of the pathogen showed large differences between the two hosts. Bacteria multiplied exponentially in the susceptible host to reach a final concentration of about 5×10^6 cells per square centimeter of leaf, compared with 5×10^4 cells in resistant plants. Resistance was decreased by high temperature, high light intensity, and short day length.

Bacterial brown spot of bean (*Phaseolus vulgaris* L.), first described in New York by Burkholder (2) in 1930, was discovered in Wisconsin in 1963 by Patel et al (11). Although once considered of minor importance (15), the disease has become serious and economically important in Wisconsin, particularly in fields with overhead irrigation (7). Transmission of the pathogen in commercial seed stocks has never been shown. In 1974, Ercolani et al (6) reported that the causal agent, *Pseudomonas syringae* van Hall, overwintered as an epiphyte on leaves of the common weed hairy vetch (*Vicia villosa*). Currently in Wisconsin, brown spot is controlled by spraying with copper fungicides (13).

A few studies have been conducted in a search for resistance to brown spot in commercial processing and dry bean varieties and in *Phaseolus* plant introductions (PI) (3,8,9). Several cultivars and PI lines showed significant disease reduction in the field but susceptible reactions in greenhouse inoculation tests. One PI line, selections of which were designated WBR 133, showed resistance to *P. syringae* in both field and greenhouse tests. Recent work (1) has identified five additional sources of resistance. Studies are under way to determine the inheritance of resistance and the allelic relationships among the different sources. Greenhouse studies with WBR 133 indicate that resistance is single-gene controlled. Certain breeding lines lacking this gene, however, show moderate to high levels of resistance in the field. Thus, other genes apparently affect resistance (S. Antonius, *personal communication*). WBR 133 has been crossed with several commercial cultivars, and a resistant bean (BBSR 130) has been released (10).

This article reports the results of experiments on the nature of resistance of WBR 133 to *P. syringae*, including possible structural barriers to penetration, multiplication of the pathogen in the susceptible and resistant hosts, and effects of environmental parameters on resistance. A preliminary report has been published (5).

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MATERIALS AND METHODS

All experiments were conducted with two *Phaseolus* lines, *P. vulgaris* 'Tenderwhite' (TW), a susceptible cultivar, and selections from the resistant WBR 133. The bacterial isolate used was *Pseudomonas syringae* Y30, isolated in 1969 by G. L. Ercolani from leaf lesions on beans grown in the area of the Hancock Experimental Farm in central Wisconsin. The cultures were maintained on NAG slants (0.8% nutrient broth, 2% glycerol, 2% agar) at 4 C and were reisolated from inoculated Tenderwhite plants once a year.

Bean seeds were sown in vermiculite and transplanted after 10 days into a soil-peat-sand mixture (3:1:1, v/v) in 13-cm clay pots and grown in the greenhouse. Plants used for growth chamber studies were grown in a peat-sand mixture (1:1) in 10-cm plastic pots and watered with Hoagland's solution.

Unless otherwise stated, plants were grown in an air-conditioned greenhouse at 22 C. Experiments were conducted in two identical growth chambers set for 12- and 16-hr days. Each experiment was repeated with the day lengths reversed to eliminate effects caused by differences in the chambers. Lighting was provided by incandescent and fluorescent bulbs and was adjusted to obtain an intensity of approximately 24,000 lux at the top of the plants. Intensities of 6,500 lux were obtained by screening the plants with cheesecloth. Temperature effects were tested in three chambers set at 20, 24, and 28 C during a 12-hr day and under 24,000 lux light intensity.

Inocula were prepared by growing the bacteria on NAG slants at 24 C for 24 hr. Bacteria were suspended in sterile distilled water, and the suspension was adjusted turbidimetrically to a concentration of 2×10^8 cells per milliliter. Leaves were inoculated by lightly spraying the underside of one-third expanded trifoliolate leaves with a paint spray gun as previously described (6); this allowed natural penetration of bacteria from the droplets on the leaf surface through the stomata and into the leaf. Because the leaf had to remain wet for approximately 20 min for adequate infection, inoculations were done before 0900 hours. This was essential for greenhouse inoculations in the summer and was used at other times of the year and in growth chamber studies so that experiments would be consistent. The inoculum concentration used resulted in infection levels on TW leaves comparable to those rated "severe" on field-grown plants. Pods at an early stage of seed development were inoculated in the same manner and also by injecting various concentrations of inoculum with a hypodermic syringe (6).

Growth of bacteria in susceptible and resistant plants was monitored at various times after inoculation by removing one 18-mm diameter disc from each of six leaflets. The discs were surface-sterilized in 10% Clorox for 1 min, then rinsed in two changes of sterile distilled water. Each group of six discs was ground in 5 ml of phosphate buffer (0.05 M, pH 6.5). Serial 10-fold dilutions were made and plated on Crosse's medium (4) (nutrient agar with 5% sucrose and 6 μg per milliliter of crystal violet). The plates were incubated at 24 C, and colonies were counted after 3–4 days.

Stomatal distributions were determined by a leaf imprint technique (14) with RTV-11 liquid silicone rubber (General Electric Co., Silicone Products Dept., Waterford, NY 12188). Leaves of greenhouse-grown plants were detached and immediately pressed into the rubber, which had just been mixed with the curing agent (Nuocure 28). After the rubber had hardened, the leaf was removed and clear nail polish was applied to the imprint. The nail polish was peeled off when dry, and stomata were counted under a microscope at ×400.

Stomatal diffusive resistance readings were taken with an L1-20S diffusive resistance sensor and measured with an L1-60 diffusive resistance meter (Lambda Electronics, Div. of Veeco Instruments Inc., Melville, NY 11746). Plants were transferred from the greenhouse to a growth chamber 1 day before measurements were begun. Light intensity at the tops of the plants was approximately 12,000 lux. Temperature was maintained at 25 ± 1 C. Initially, readings were taken three times a day, but when no significant differences were found, readings were taken daily on the upper and lower surfaces of trifoliolate leaflets, starting when they were about one-fourth to one-third expanded (5–7 cm long) and continuing for 6 days until the leaves were fully expanded.

Tissue for light microscope sections was prepared as for transmission electron microscopy. Leaf pieces were fixed in 5% glutaraldehyde and Palade's osmium, stained in saturated uranyl acetate, dehydrated in an acetone series, and embedded in Spurr's medium. Sections were cut approximately 0.5 μm thick, dried onto glass slides, mounted with Permount (Fisher Scientific Co., Fair Lawn, NJ 07410), and viewed under phase contrast.

Leaves prepared for scanning electron microscopy were cut into 2-mm^2 pieces and fixed in 5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.1, for 24 hr at 4 C. The tissue pieces were washed in four changes of distilled water and frozen in Freon cooled in liquid nitrogen for 15–20 sec. The sections were placed in tubes, precooled

TABLE 1. Stomatal diffusive resistance of leaves of Tenderwhite and of WBR 133[y]

Host	Leaf size (cm)	Stomatal resistance (sec/cm)[z] Lower leaf surface	Upper leaf surface
Tenderwhite	5–8	2.5 a	18.9 c
Tenderwhite	9–12	2.5 a	25.2 d
WBR 133	5–7	2.25 a	14.3 c
WBR 133	8–10	3.3 a	10.6 b

[y]Readings were taken with a diffusive resistance porometer on leaves 5–8 cm long (size used for inoculation) and on more expanded leaves. Resistance values for the resistant WBR 133 are not significantly higher than those for the susceptible Tenderwhite.
[z]Same letter denotes no significant differences ($P = 0.05$).

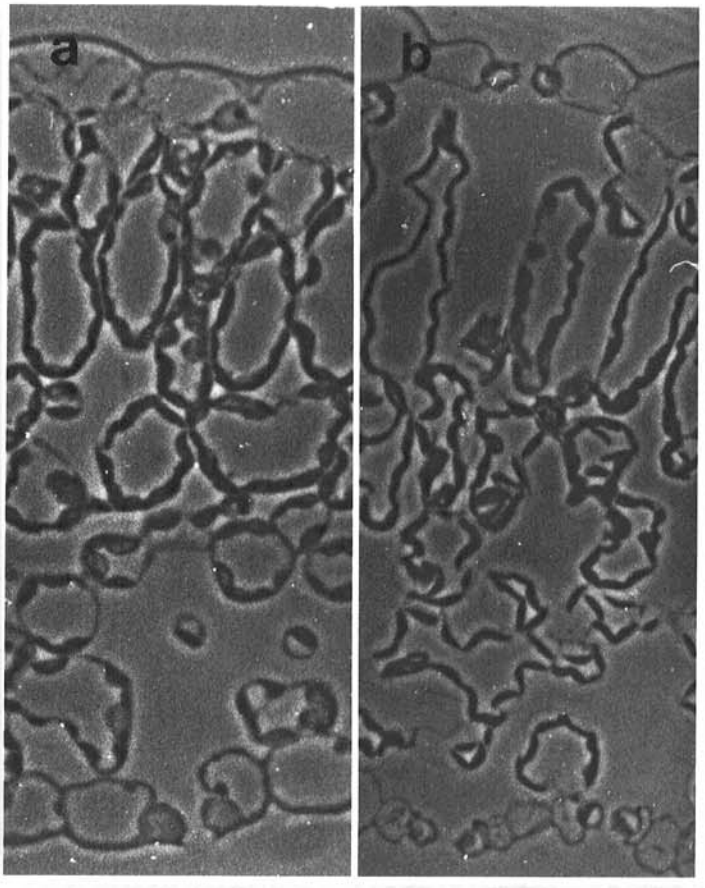

Fig. 1. Cross section of trifoliolate leaf of **a,** Tenderwhite (×500) and **b,** WBR 133 (×400) viewed under phase microscopy. Note similarity between stomata.

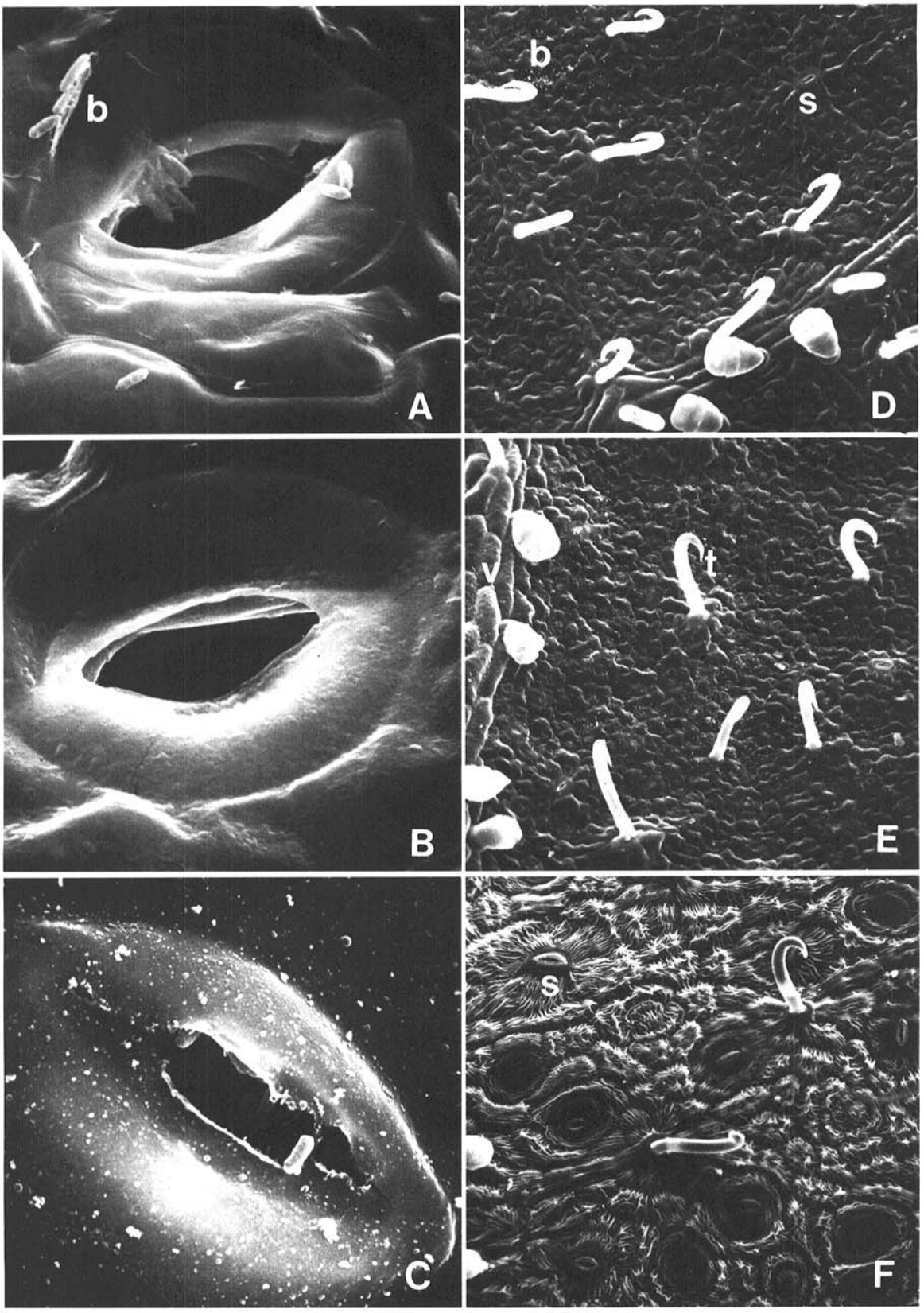

Fig. 2. Scanning electron microscope pictures of inoculated surfaces of Tenderwhite (TW) and WBR 133 trifoliolate leaves and pods. Micrographs of stomata on **A**, lower surface of TW leaf, **B**, lower surface of WBR 133 leaf, and **C**, WBR 133 pod. Note size of bacteria in relation to stomatal aperture. (A and B, ×4,400; C, ×5,300) Surface structure of **D**, TW lower leaf, **E**, WBR 133 lower leaf, and **F**, WBR 133 pod. Leaf and pod surface structures appeared similar in the two plant lines. (D–F, ×200) b = bacteria, v = vein, t = trichome, s = stomata.

in dry ice, and lyophilized for 18 hr. Pod tissue was fixed and washed in the same manner. Sections were dehydrated in an alcohol series (30–100%) followed by several changes in amyl acetate, then dried in a Denton critical point drying apparatus using liquid CO_2. Both leaves and pods were mounted on aluminum stubs with silver conducting paint, coated with gold palladium, and viewed in a JELCO JS14-U3 scanning electron microscope.

RESULTS

Symptoms. Inoculation of susceptible leaves resulted in many small water-soaked lesions surrounded by narrow yellow margins. Puckering of the tissue around the lesions also occurred. Lesions on resistant leaves were smaller, dry rather than water-soaked, and fewer in number but were also surrounded by narrow yellow bands. Lesions appeared in plants of both lines 48 hr after inoculation. At this time, individual lesions on susceptible leaves contained an average of 9.0×10^4 bacterial cells and those on resistant leaves, 2.5×10^3 cells. Bacterial populations in lesions remained constant up to 5 days after inoculation. Spray inoculation of susceptible pods resulted in large, sunken, water-soaked lesions. Resistant pods showed almost no symptoms under the same conditions; occasionally, a single necrotic spot was seen. Pods were also injected with inoculum concentrations of 10^7, 10^5, and 10^3 cells per milliliter. Symptoms appeared on susceptible pods in about 2 days. With the highest concentration, the inoculated area became sunken and water-soaked; the area later enlarged and occasionally produced a bacterial exudate. With the lower concentrations, small water-soaked lesions appeared, enlarged, and eventually coalesced. In resistant pods, the 10^7 inoculum resulted in browning of the inoculated area within 24 hr; the area later became sunken and water-soaked but did not enlarge or produce a bacterial exudate. The 10^5 concentration resulted in pinpoint necrotic lesions. At 10^3 cells per milliliter, no symptoms were observed.

Structural barriers to penetration. The possible role of stomata as barriers to pathogen penetration in WBR 133 was investigated using four techniques: (i) histologic sections, (ii) leaf surface imprints, (iii) diffusive resistance porometer readings, and (iv) scanning electron microscopy.

Histologic sections. Cross sections of leaves from TW and WBR 133 are shown in Fig. 1. Stomatal structure appeared similar in the two plants.

Leaf imprints. Imprints were made of the lower surface of 15 and the upper surface of five trifoliolate leaflets for each of the plant lines. Twenty microscopic views of each of three different areas on each leaf imprint were counted and the values averaged. No significant differences ($P = 0.05$) were found between the susceptible and resistant hosts in stomatal numbers on the lower leaf surface; WBR 133 plants averaged 5.55×10^4 stomata per square centimeter of leaf surface and TW plants, 5.70×10^4. On the upper leaf surface of both plant lines, stomata were clustered in very high numbers along the major veins. WBR 133 plants also had stomata on the leaf lamina at a concentration of 4.5×10^3 per square centimeter; these were lacking on the upper surface of TW leaves.

Diffusive resistance porometer readings. Results are shown in Table 1 and are averages of eight experiments, each utilizing six plants of each line. No significant differences were found in stomatal resistance readings of the lower surfaces of leaves 5–8 cm long (inoculation size) or more expanded leaves. The upper surface of WBR 133 leaves had lower stomatal resistance values than those of TW leaves, probably because of differences in stomatal numbers. Because individual WBR 133 plants varied in disease resistance under greenhouse conditions, we also investigated stomatal resistance and degree of disease resistance of leaves on individual plants. We were unable to correlate these two factors.

Scanning electron microscopy. Scanning electron microscopic pictures of inoculated leaf and pod surfaces showed no differences in stomatal structure that would appear to affect penetration of bacteria (Fig. 2 A–C). Bacteria often could be seen on the edges of guard cells and within the substomatal chamber. Stomatal apertures were so large in relation to bacteria that even small openings were sufficient for penetration. Such surface features as structure and location of veins and trichomes and the relation of stomata to surrounding epidermal cells appeared to be very similar in the two plant lines (Fig. 2 D–F).

Multiplication of the pathogen in susceptible and resistant leaves. Multiplication of *P. syringae* in the leaves of TW and WBR 133 is shown in Fig. 3. The results are averages of eight inoculation experiments carried out between April and September 1975. Lightly spraying leaves with an inoculum concentration of 2×10^8 cells per milliliter resulted in an initial concentration of about 10^4 cells per square centimeter of leaf tissue. In TW leaves, bacteria multiplied exponentially, reaching a maximum concentration of

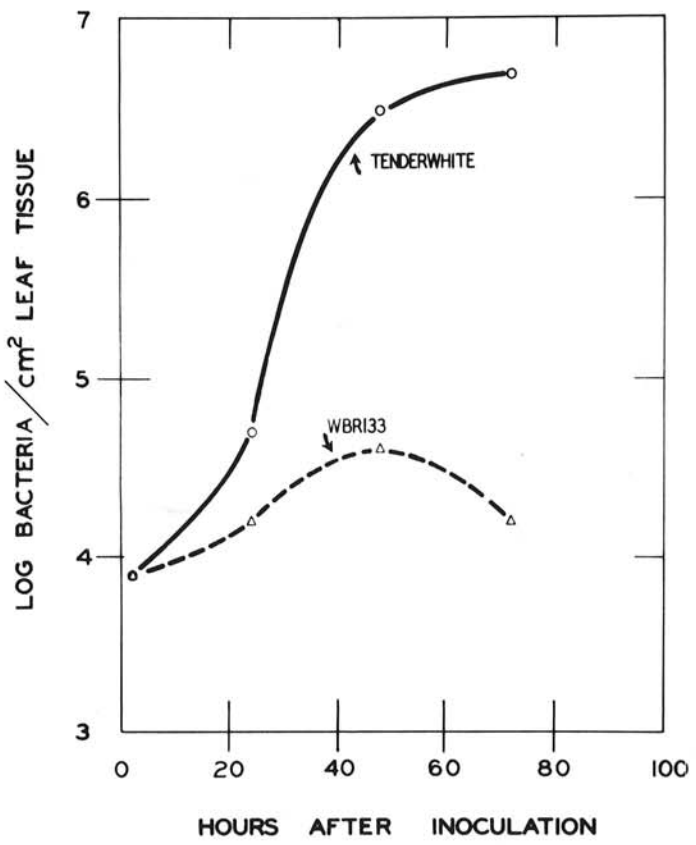

Fig. 3. Multiplication of *Pseudomonas syringae* in leaves of Tenderwhite and WBR 133. Lesions developed 48 hr after inoculation.

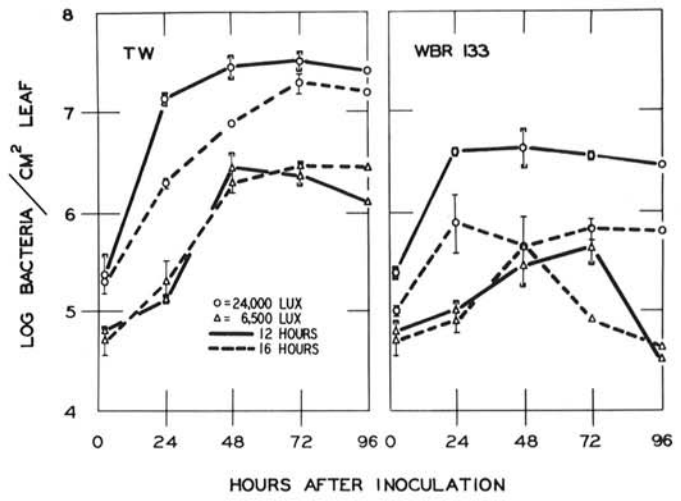

Fig. 4. Multiplication of *Pseudomonas syringae* in Tenderwhite (TW) and WBR 133 leaves under controlled-environment conditions. All plants were grown at 24 C. Lesions appeared 48 hr after inoculation.

about 5×10^6 cells per square centimeter 48 hr after inoculation. This peak point corresponded with the time of lesion development. In WBR 133 leaves, pathogen growth was much slower and reached a maximum population of only about 5×10^4 cells per square centimeter. No significant changes in bacterial populations occurred after 48 hr in either plant line.

Environmental effects on resistance stability. Resistance of WBR 133 plants was not stable under greenhouse conditions, and a serious breakdown in resistance occurred in the fall of 1975. Because pathogen changes did not appear to be the cause, we initiated studies on the effect of environmental parameters on resistance.

Controlled experiments in growth chambers showed that temperature, light intensity, and day length affected disease reaction. Effects of the interaction of day length and light intensity on bacterial multiplication are shown in Fig. 4. Initial inoculum dose of bacteria was generally much greater in the growth chambers than in the greenhouse, although final bacterial populations in plants at low light intensities were similar to those obtained in the greenhouse. Initial counts of bacteria from inoculated TW plants were higher at high light intensities than at low, indicating that more bacteria were able to enter the leaf at high intensities. In addition, final bacterial populations were greater and lesions were more numerous on TW plants grown at high light intensities. Inoculum dose and bacterial multiplication were not significantly different in WBR 133 plants grown at low and at high light intensities during a 16-hr day but were significantly greater in WBR 133 plants grown under high light intensities during a 12-hr day. In TW plants, differences in day length appeared to affect bacterial multiplication at high light intensities but not at low, as multiplication at 24,000 lux was greater during a 12-hr day than during a 16-hr day. Bacterial multiplication in TW plants was greater with increasing temperature from 20 to 28 C (Fig. 5). The pattern in WBR 133 plants was the same except at 28 C, when initial inoculum dose and growth of bacteria were reduced. At this temperature, WBR 133 plants, which are large and easily water stressed, were somewhat wilted even though the soil was kept moist. Increased watering of WBR 133 plants in the greenhouse resulted in higher multiplication of bacteria (*unpublished*). Water stress presumably was causing the decrease in multiplication.

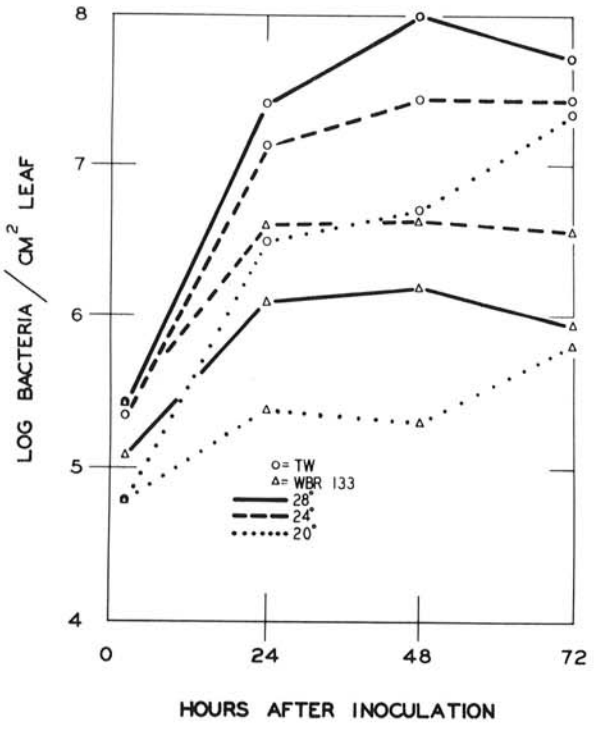

Fig. 5. Multiplication of *Pseudomonas syringae* in Tenderwhite (TW) and WBR 133 leaves grown at 20, 24, or 28 C with 24,000 lux and 12-hr day length. Lesions appeared 48 hr after inoculation.

DISCUSSION

WBR 133 leaves and pods showed a high degree of resistance to brown spot. Lesions on WBR 133 plants were smaller and fewer in number than those on TW plants. This difference in symptoms was reflected in the large difference in pathogen multiplication in the susceptible and resistant host plants.

P. syringae is disseminated by windblown rain and penetrates the host through the stomata. Stomatal structure and behavior have been implicated in resistance to several diseases (12), but our studies indicate that stomata are probably not serving as a line of defense in WBR 133. We found no significant differences in stomatal size, numbers, or structure between the two hosts. This is substantiated by the similar initial bacterial multiplication in TW and WBR 133. The possibility that stomata play a role in disease development is suggested by the high initial inoculum dose in plants grown in the growth chamber, particularly those grown at high light intensities. The increase in inoculum dose in WBR 133 plants grown during a 12-hr day and under high light intensity appears to be a significant factor in the breakdown of resistance under these conditions, and a change in stomatal behavior possibly is affecting this. Unfortunately, porometer readings were not taken before inoculation of these plants. Differences in leaf surface features, such as location and structure of veins and trichomes, and the relationship of the stomata to surrounding epidermal cells, which could play a role in resistance by affecting retention of inoculum drops and movement of bacteria into the stomata, do not appear to be factors in the difference in brown spot reaction of the two cultivars.

Growth chamber studies indicated that high temperatures, high light intensity, and short day length all increased disease development in both TW and WBR 133 plants. The combined effect of high light intensity and short day length in reducing resistance was particularly pronounced in WBR 133 plants, in which both inoculum dose and bacterial multiplication were increased. Similar conditions presumably were causing the breakdown in resistance we observed in the fall of 1975. This obviously presents no problem for utilization of resistant lines in the field but does point out the necessity of controlling light conditions when lines are being screened in the greenhouse.

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