Growth Kinetics and Interactions of *Pseudomonas syringae* with Susceptible and Resistant Bean Tissues

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ABSTRACT

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Differences were found in symptom expression and in multiplication rates of *Pseudomonas syringae* isolate Y-30 in leaves and pods of bean plants susceptible (cultivar Tenderwhite) and resistant (WBR 133) to bacterial brown spot of bean. Symptom expression in the two hosts was different at all inoculum concentrations tested, but there were almost no differences in bacterial growth rates and final bacterial populations in the two hosts at high inoculum levels. In pods that received low inoculum levels, *P. syringae* multiplied more slowly in WBR 133 than in Tenderwhite. In leaves, rates of *P. syringae* multiplication were the same during the exponential growth phase (0–12 hr after inoculation), but were much slower in WBR 133 than in Tenderwhite during the transition stage between exponential growth and the stationary phase. Doubling times of *P. syringae* in Tenderwhite were not affected by inoculum concentration. Infiltration of Tenderwhite leaves and pods with the incompatible pathogen *Pseudomonas coronafaciens* resulted in rapid necrosis of the inoculated area, a symptom characteristic of a hypersensitive response. However, cessation of growth of *P. coronafaciens* was not correlated with the development of visible necrosis. In leaves, *P. coronafaciens* showed an 8–12 hr lag phase before starting to multiply. Doubling times of *P. coronafaciens* in both leaves and pods were longer than those of *P. syringae* in leaves and pods of either host, unless high inoculum concentrations were used. Doubling times of *P. coronafaciens* decreased with increasing inoculum concentration. Electron microscopy of pod tissue in the three host-pathogen combinations showed changes associated with an apparent defense reaction by the host in the incompatible interactions. A fibrillar material, which appeared to arise from the host cell wall, enveloped bacteria in the intercellular spaces. However, this defense reaction did not appear effective, and, within 8 hr after inoculation, bacteria were multiplying and filling the intercellular spaces. In leaves, envelopment of bacteria by fibrillar material was seen only rarely and could be found in all three host-pathogen combinations.

Additional key words: bacterial brown spot of bean, electron microscopy, hypersensitivity, disease resistance.

The physiological basis for the resistance of plants to bacterial multiplication is not well understood. Host plants inoculated with incompatible bacteria often give a hypersensitive reaction (HR), characterized by rapid collapse of host cells and localization of the pathogen (12). Studies on the multiplication of bacteria in bean plants (8,13,14) have shown that both compatible and incompatible bacteria can multiply in beans, but at different rates and to different final populations. Multiplication of incompatible bacterial (*Pseudomonas morsprunorum* and a lilac strain of *P. syringae*) stopped at 16–24 hr (13,14) or 2–3 days (8) after inoculation, which corresponded to the development of a visible HR. A compatible bacterium (*P. phaseolicola*), by contrast, continued to multiply until 3–5 days after inoculation when typical disease symptoms appeared. Differences in the inoculum dose had little effect on the generation time of the compatible bacteria, but growth ceased earlier at higher doses (8). Inoculum dose affected the generation time of incompatible bacteria, but not the time of appearance of the HR (8,13). Final bacterial populations always were less in the incompatible than in the compatible reaction. No visible HR developed when the inoculum dose was less than 10^4 cells per milliliter in pods (13), or 10^6–10^7 cells per milliliter in leaves (8,14), although microscopic studies showed scattered groups of dead host cells in leaf mesophyll tissue with an inoculum dose of 2×10^5 cells per milliliter (14). No symptoms could be seen when plants were inoculated with saprophytic bacteria. Lyon and Wood (14) found that the HR in bean leaves was not affected by light or humidity, would occur at 37 C and had a 60–90 min induction time. These results are somewhat different from what was reported for tobacco (16).

Attempts to induce the HR with products from HR-inducing bacteria have not been successful (14,16). Recent evidence suggests that attachment of bacterial cells to host cell walls may be an initial step in the induction of an HR. In tobacco (10,17) and cotton (2), incompatible bacteria are reported to attach readily to host cell walls, followed by envelopment from material arising from the host cells. A similar study with beans (19) reported that only

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saprophytes were enveloped, although later work (1) indicated that this phenomenon was not an active response by the host.

This paper reports the kinetics of *P. syringae* multiplication in plants with bean lines susceptible (cultivar Tenderwhite) and resistant (WBR 133) to bacterial brown spot of bean, and the reaction of the lines to bacterial multiplication. These reactions were compared to the changes that occur in Tenderwhite beans when inoculated with incompatible pathogen *P. coronafaciens*. This research was conducted to determine if the attachment and envelopment response reported to occur in other hosts was active in the resistance of WBR 133 to *P. syringae*. Because *P. syringae* does not induce a supersensitive reaction on WBR 133 (7), the hypersensitive combination (*P. coronafaciens* on Tenderwhite) was included for comparison. Preliminary reports have been published (5,6).

MATERIALS AND METHODS

The bacterial isolates used were *P. syringae* Y-30, isolated in 1969 by G. L. Ercolani from lesions on beans grown at the University of Wisconsin's Hancock Experimental Farm in central Wisconsin, and *P. coronafaciens* PC-27, provided by M. P. Starr (Bacteriology Department, University of California, Davis). *P. coronafaciens* was chosen because it produced rapid necrotic symptoms when introduced at varying inoculum concentrations into Tenderwhite leaves and pods. The cultures were maintained on NAG (0.8% nutrient broth, 2.0% glycerol, 2.0% agar) slants at 4 C, and were reisolated from their respective hosts (oat or bean) periodically.

Bean seeds were sown in vermiculite, and seedlings were transplanted after 10 days into a soil:peat:sand (3:1:1, v/v) mixture in 13-cm-diameter clay pots and grown in the greenhouse. WBR 133 requires short days for flowering; for pod production WBR 133 plants, 2–3 wk after transplanting, were placed in a growth chamber under an 8-hr light period per day. Plants flowered in about 3 wk and then were transferred back to the greenhouse.

Inocula were prepared by suspending bacteria grown on NAG slants at 24 C for 24 hr in water. For bacterial multiplication studies, the suspension was adjusted turbidimetrically to a concentration of 2×10^8 cells per milliliter, and tenfold dilutions were made from this suspension. For higher inoculum concentrations (10^9 cells per milliliter) the bacteria were washed twice in water before the inoculum suspensions were prepared.

Pods were picked at an early stage of seed development, washed in running water for 10 min, and then injected (hypodermic syringe) with a bacterial suspension at five to six sites on each pod. Pods were rinsed in water and incubated at room temperature in a glass-covered tray lined with moistened filter paper. Humidified air was passed through the chamber.

One-third-expanded trifoliolate leaves were used because older leaves are less susceptible to bacterial invasion. All plants were inoculated and incubated in the greenhouse. For bacterial multiplication studies, leaves were inoculated by placing a piece of foil with a 12-mm-diameter hole over the abaxial surface of the leaf. Bacterial suspensions of varying concentrations (10^4–10^8 cells per

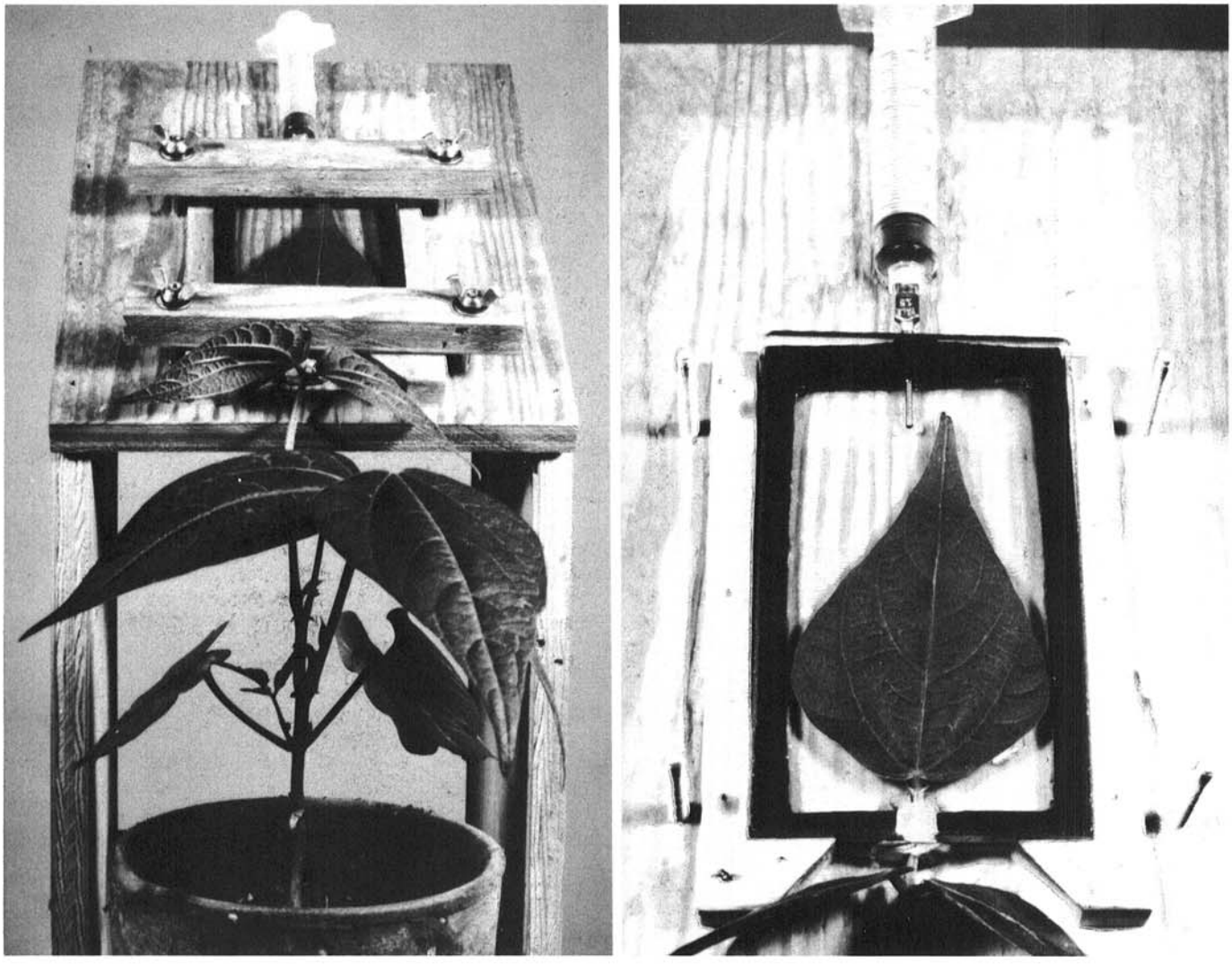


Fig. 1. Device for vacuum infiltration of bacterial suspensions into leaves being prepared for electron microscopy.

milliliter) were sprayed with a paint spray gun (9) onto the leaf until the exposed area became watersoaked.

For electron microscopy, leaves were vacuum infiltrated with a bacterial suspension containing 10^9 cells per milliliter in a small vacuum chamber (Fig. 1). The inoculating chamber consisted of two glass plates (75 × 128 mm) with a neoprene rubber seal (3 mm thick). The petiole of a leaflet was placed through an opening in the seal at the base of the chamber, and the opening was sealed with modeling clay. The top glass plate was clamped down and inoculum was added with a syringe through the 1.83-mm-diameter (15-gauge) needle at the top of the chamber. Ten milliliters of inoculum was sufficient to fill the chamber three-quarters full. The syringe was removed, and a vacuum hose was attached to the needle. A 0.692 kg/cm^2 (10-lb) vacuum was applied for 1 min and then released quickly. The process was repeated until the leaf was almost totally infiltrated (usually twice). The infiltrated areas were marked. Because these leaves contained so much inoculum, they tended to collapse and dry out very quickly. To alleviate this problem, plants were covered with plastic bags after all water-soaking had disappeared (20–30 min after inoculation). Bags were removed after 12 hr. Leaves infiltrated with water showed no visible signs of damage 24 hr after infiltration.

Bacterial populations in leaves were monitored by removing six 18-mm-diameter disks from the inoculated area of six different leaves at 0, 4, 8, 12, 24, 48, 72, and 96 hr after inoculation. These disks were surface sterilized in 10% Clorox for 1 min and rinsed in two changes of sterile distilled water. Pod tissue was sampled by cutting six 7-mm-diameter disks from the infiltrated areas of six different pods at 0, 6, 12, 24, 48, and 72 hr after inoculation. Disks were cut to the seed cavity. Each group of six disks was ground in 5 ml of phosphate buffer (0.05 M, pH 6.5). Serial 10-fold dilutions were plated on Crosse's medium (4). Plates were incubated at 24 C, and colonies were counted after 3–4 days. Multiplication studies were repeated three times with each host-pathogen combination at each inoculum level. The data reported are the means of the three trials.

For electron microscopy, samples of inoculated tissue were obtained at 0, 2, 4, 8, 12, 24, and 48 hr after infiltration. The samples were fixed under vacuum with 5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4. After fixation, the tissue was rinsed in cacodylate buffer, postfixed in Palade's fixative, stained with saturated uranyl acetate, dehydrated in an acetone series (30–100%), and embedded in Spurr's medium. Sections were mounted on collodion-covered grids, stained with lead citrate, and viewed in a JEM-7 transmission electron microscope.

RESULTS

The three bacteria-host combinations gave different symptoms on both bean leaves and pods. Inoculation of Tenderwhite pods with high concentrations (10^7–10^9 cells per milliliter) of *P. syringae* (susceptible combination) resulted in water-soaking of the infiltrated areas at 24 hr after inoculation. These areas enlarged and eventually produced a bacterial exudate. At lower inoculum concentrations (10^3–10^5 cells per milliliter), small water-soaked spots appeared after 36–48 hr. These spots enlarged and eventually coalesced. In the resistant combination (WBR 133 inoculated with *P. syringae*), high inoculum doses caused an initial browning of the inoculated area, which later became water-soaked, but this did not enlarge or produce a bacterial exudate. Low inoculum concentrations resulted in either small necrotic spots or no symptoms. Time required for symptom development was the same as in the susceptible combination. At high inoculum concentrations (10^7–10^9 cells per milliliter) in the hypersensitive combination (Tenderwhite inoculated with *P. coronafaciens*), the infiltrated area became necrotic 8–12 hr after inoculation. By 48–72 hr, the necrotic area appeared sunken. At low inoculum concentrations (10^3–10^5 cells per milliliter), scattered necrotic spots appeared at 24–48 hr after inoculation. At 10^5 cells per milliliter, more necrotic spots developed and in some cases, the spots had coalesced within 72 hr. Symptoms did not extend beyond the infiltrated area at any

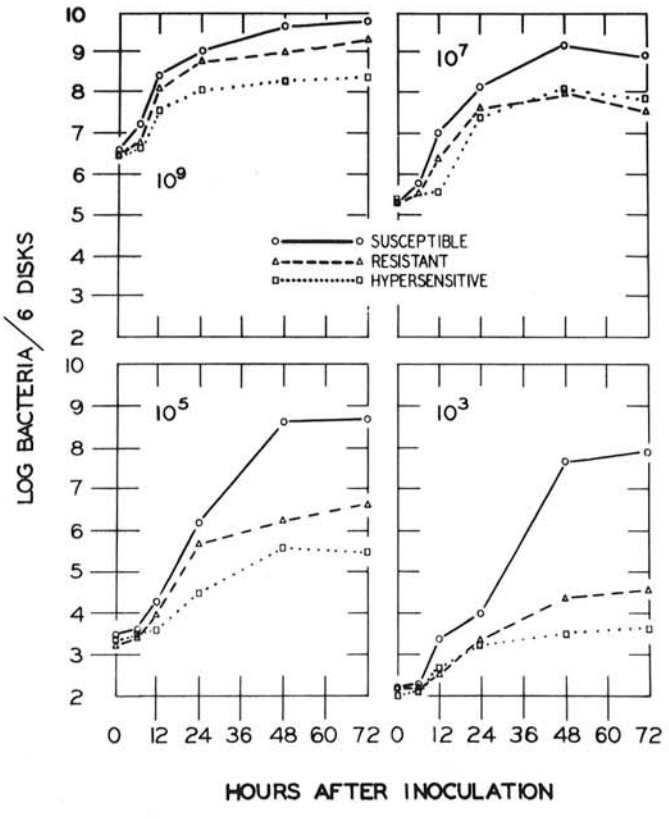


Fig. 2. Multiplication of bacteria in bean pods in the susceptible (*P. syringae* in Tenderwhite), resistant (*P. syringae* in WBR 133), and hypersensitive (*P. coronafaciens* in Tenderwhite) combinations at four different inoculum concentrations (10^9, 10^7, 10^5, and 10^3 cells per milliliter).

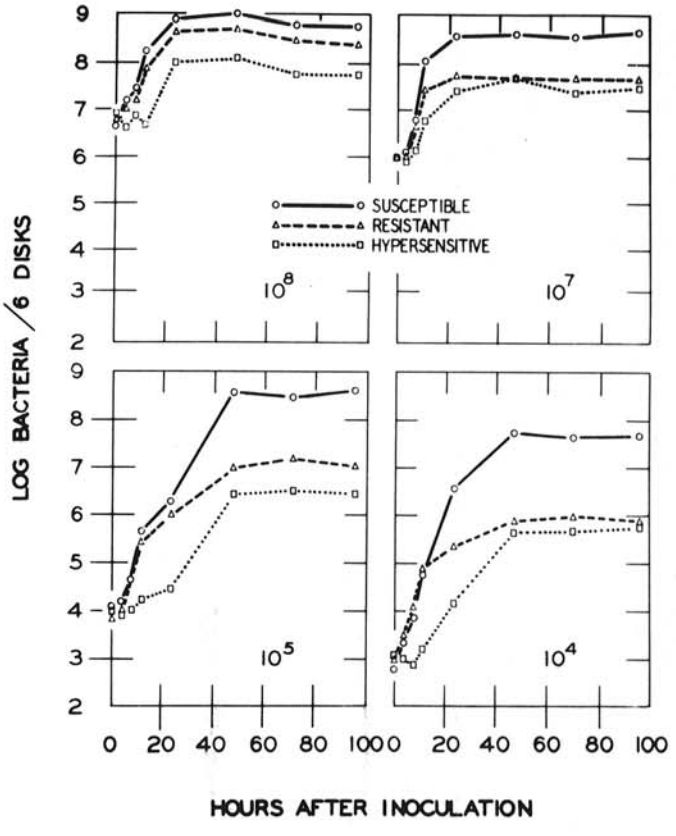


Fig. 3. Multiplication of bacteria in bean leaves in the susceptible (*P. syringae* in cultivar Tenderwhite), resistant (*P. syringae* in WBR 133), and hypersensitive (*P. coronafaciens* in Tenderwhite) combinations at four different inoculum concentrations (10^8, 10^7, 10^5, 10^4 cells per milliliter).

inoculum concentration.

When leaves were vacuum infiltrated with high concentrations of inoculum (10^6–10^9 cells per milliliter) in the susceptible combination, the infiltrated area became water-soaked 24 hr after inoculation. By 48 hr, the leaves had collapsed and many of the petioles were shrivelled. With low inoculum concentrations (10^4–10^5 cells per milliliter), many tiny confluent lesions appeared on the leaf about 48 hr after inoculation. By 72 hr, the leaves had collapsed. In the resistant combination, high inoculum concentrations resulted in dessication and collapse without necrosis of the infiltrated tissues by 24 hr. Symptoms did not progress beyond that time. No symptoms were visible in the resistant combination when low inoculum concentrations were used. In the hypersensitive combination, high inoculum concentrations resulted in necrosis of the infiltrated area at 12 hr after inoculation. At low inoculum concentrations, small necrotic flecks appeared around 24 hr. Symptoms did not progress beyond those times.

When bacteria were infiltrated into pods in the three host-bacteria combinations, there was an initial period of slow growth (0–6 hr) followed by a period of rapid exponential growth (Fig. 2). At low inoculum concentrations, the period of exponential growth continued until approximately 48 hr after inoculation; however, at high inoculum concentrations, this period was shorter. In most cases the bacteria started to multiply at a slower rate at approximately 12 hr and finally entered the stationary phase at about 48 hr.

At low inoculum concentrations, there were large differences in the doubling times of exponentially multiplying bacteria in the three combinations. At 10^3 cells per milliliter, the doubling time of bacteria in the hypersensitive combination was approximately 9.7 hr versus 5.7 hr for bacteria in the resistant combination and 2.3 hr for bacteria in the susceptible combination. Differences between doubling times became less as the inoculum concentration was increased. At 10^9 cells per milliliter, there were almost no differences in doubling times between bacteria in the three combinations, with doubling times of 1.5, 1.5, and 2.0 hr for bacteria in the susceptible, resistant, and hypersensitive combinations, respectively. The doubling time of *P. coronafaciens* decreased with increasing inoculum dose (9.7, 4.0, 2.0, and 2.0 hr for 10^3, 10^5, 10^7, and 10^9 cells per milliliter, respectively), whereas that of *P. syringae* remained fairly constant except for bacteria inoculated into WBR 133 at very low concentrations in which case the doubling times were considerably longer. Differences in final bacterial populations between the susceptible combination and the resistant and hypersensitive combinations were large at low inoculum doses, but slight at high inoculum doses. Differences at high inoculum doses appeared to be due as much to differences in growth rate during the 6-hr slower growth period as to differences in growth rate during the exponential phase.

When *P. syringae* was infiltrated into leaves of the susceptible and resistant host, little or no lag period was seen (Fig. 3). Bacteria multiplied exponentially until approximately 12 hr. Doubling times of bacteria in the two hosts were similar (average of 1.6 hr in Tenderwhite, 1.8 hr in WBR 133), and they remained constant at all inoculum levels. After 12 hr, however, there was a large reduction in the growth rate of the bacteria in the resistant host compared to that in the susceptible host at all inoculum concentrations except the highest. Doubling times during the transition phase between the exponential growth phase and the stationary phase (12–48 hr at 10^4 and 10^5 cells per milliliter; 12–24 hr at 10^7 cells per milliliter) averaged 4.2 hr in the susceptible host, compare to 10.0 hr in the resistant.

In contrast, a definite lag period was seen in the multiplication of *P. coronafaciens*. The exponential growth period did not begin until 8–12 hr after inoculation. Bacteria entered the stationary phase at the same time as *P. syringae*, 24 hr at high and 48 hr at low inoculum doses. Exponential-phase doubling times of *P. coronafaciens* were longer than those of *P. syringae*, and showed the same trend as seen in pods of decreased doubling times with increased inoculum dose (doubling times were 4.3, 3.4, 2.7, and 2.8 hr at 10^4, 10^5, 10^7, and 10^8 cells per milliliter, respectively).

In leaves, there was no reduction in stationary phase populations in any of the combinations for up to 7 days.

In pods (Figs. 4–7), within 2 hr after inoculation there were changes in the resistant and hypersensitive combinations that seemed to indicate the induction of a defense mechanism by the host. A fibrillar substance, which appeared to arise from the host cell walls, had enveloped some of the bacteria (Fig. 4). The host cell wall occasionally appeared to be eroded at the point of bacterial attachment (Fig. 5 [arrows]). The thin fibrillar outer layer of the host cell wall (pellicle) often was seen extending across the intercellular space and enveloping bacteria (Fig. 5). Eighty-four percent and 73% of the micrographs for the resistant and for the hypersensitive combinations, respectively, showed signs of bacterial attachment and/or envelopment. In contrast, most bacteria in the susceptible combination remained free (Fig. 6). Fibrillar material partially surrounding some bacteria was seen in 27% of the micrographs of the susceptible combination. Envelopment of bacteria by the pellicle was never seen in the susceptible combination.

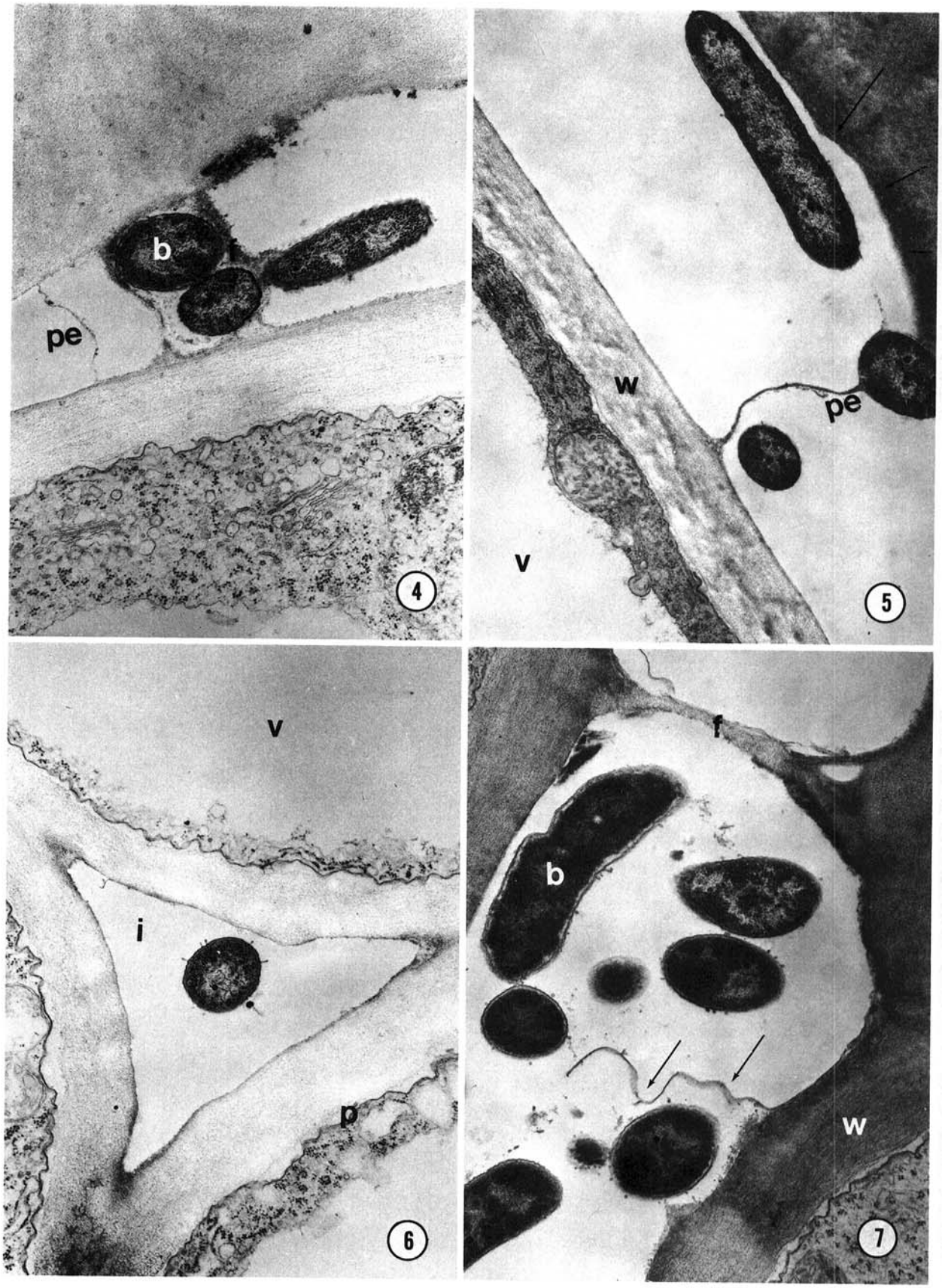
Beyond 2 hr, however, there appeared to be no progression of the envelopment response (Fig. 7). Fibrillar material could be seen extending into the intercellular spaces, and some bacteria appeared to have been immobilized, but most bacteria were dividing freely. Cell collapse occurred in all three combinations at 12–24 hr after inoculation. Bacteria appeared normal at this time.

In leaves (Figs. 8–12), almost no signs of cell wall envelopment of bacteria could be seen at any stage after inoculation in any of the three combinations (Figs. 8–10). Occasionally, some bacteria were enveloped (Fig. 11) and there was a separation of the plasmalemma from the cell wall and an accumulation of vesicles in the space between them. Such a response was not seen in pods, but has been reported in other host-parasite systems (2,10,17). As in pods, host cell collapse in leaves occurred in all three combinations 12–24 hr after inoculation (Fig. 12). At this time, bacteria filled the intercellular spaces and were surrounded by a matrix. In some instances this matrix appeared to be fibrillar; in others, it was very dense and appeared to be somewhat impermeable to the embedding medium. It is not known whether this matrix is of host or pathogen origin.

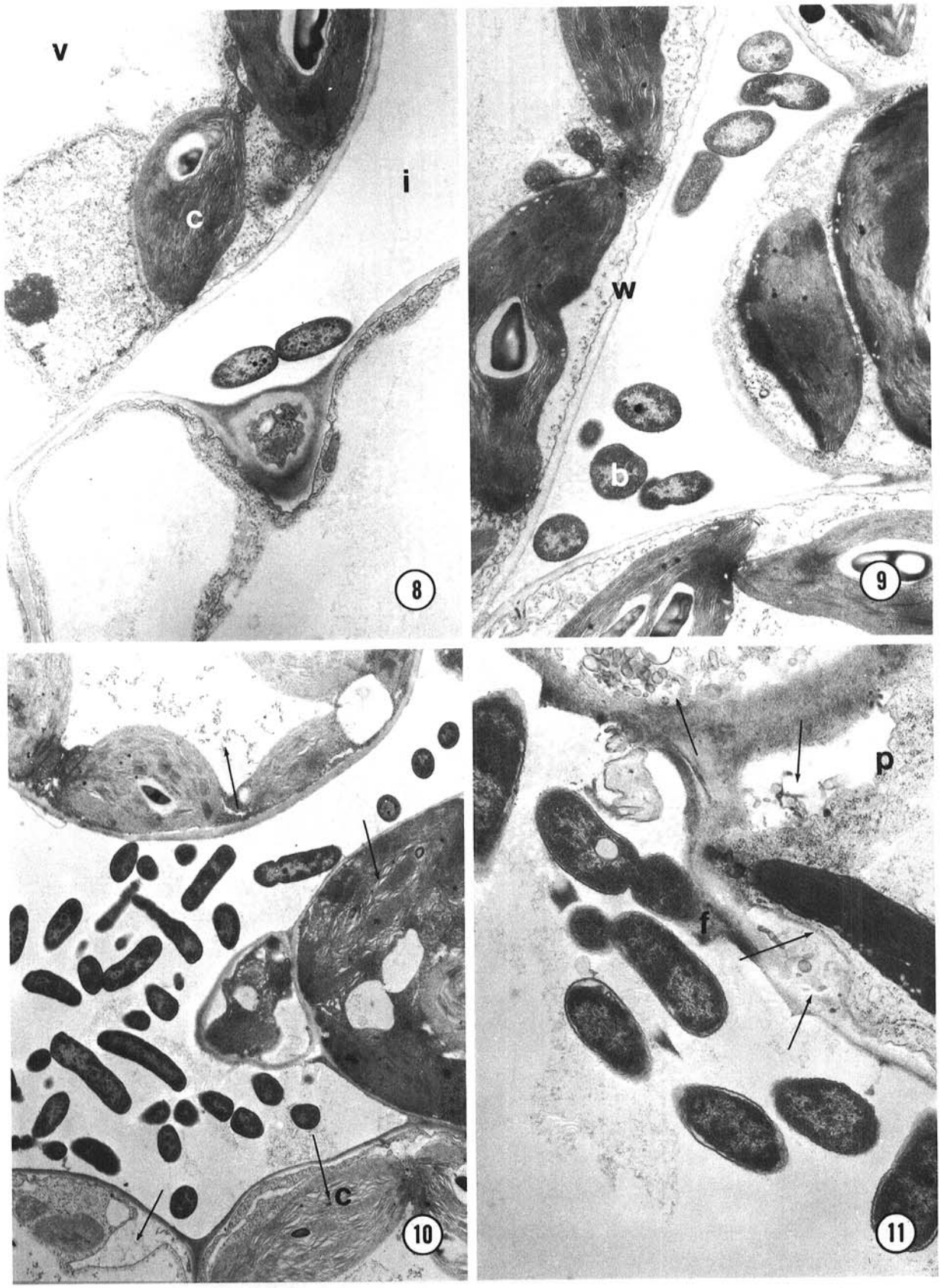
DISCUSSION

Differences were seen in symptom expression, time of symptom development, and multiplication of bacteria in the three host-bacteria combinations that were studied. In pods, *P. syringae* multiplied at a slower rate in the resistant host than in the susceptible host, unless high inoculum concentrations were used. In leaves, *P. syringae* multiplied at the same rate in the susceptible and in the resistant hosts during the exponential growth phase, but at a much slower rate in the resistant compared to the susceptible host during the transition stage between the exponential growth phase and the stationary phase. As in pods, differences were slight at high inoculum doses. Duration of exponential growth and time to reach the stationary phase were the same in the two hosts at the same inoculum level. Thus, resistance of WBR 133 appears to be due to differences in the rate of *P. syringae* multiplication, and not in the duration of the growth phase. These results are in contrast with other reports on bacterial multiplication (3,15,20) in which the duration of the pathogen growth phase was considerably shorter in resistant hosts than in susceptible hosts.

Infiltration of leaves and pods with *P. coronafaciens* resulted in rapid necrosis of the inoculated area, a symptom characteristic of a hypersensitive reaction (12). Bacterial growth curves, however, were different from those reported for other systems (14,18,20). There was no correlation between the appearance of visible necrosis and the cessation of growth of *P. coronafaciens*; necrosis was visible at 12–24 hr after inoculation, but bacterial growth ceased at the same time as in the susceptible and resistant combinations (24–48 hr). Thus, resistance of Tenderwhite beans to *P. coronafaciens* was not due to an abrupt termination of growth, but to a slower rate of growth, and in leaves, to a delayed and thus shorter exponential growth period. The doubling time of *P.*



Figs. 4–7. Electron micrographs of bean pod tissue inoculated with bacteria. **4,** Hypersensitive combination (*Pseudomonas syringae*/Tenderwhite bean) as seen 2 hr after inoculation. Note fibrillar material (f) surrounding the bacteria (b) in the intercellular space. The pellicle (pe) has lifted off the host cell wall and extends across the intercellular space. ×22,950. **5,** Resistant combination (*P. syringae*/WBR 133 bean) as seen 2 hr after inoculation. Cell wall (w) appears eroded where it is in contact with the bacteria (arrows). Note pellicle (pe) enveloping a bacterium. v=vacuole. ×21,600. **6,** Susceptible combination (*P. syringae*/Tenderwhite bean). The bacterium is free in the intercellular space (i). p=plasmalemma. ×22,500. **7,** Hypersensitive combination (*P. coronafaciens*/Tenderwhite bean) as seen 8 hr after inoculation. Note fibrillar material (f) extending across the intercellular space and the apparent envelopment of the bacterium (b) by the pellicle (arrow). Most bacteria, however, are multiplying and appear normal. Similar occurrences are seen in the resistant reaction. w=cell wall. ×23,400.



Figs. 8–11. Electron micrographs of bean leaf tissue inoculated by vacuum infiltration with bacterial suspensions. **8,** The susceptible combination (*Pseudomonas syringae*/Tenderwhite bean) as seen 2 hr after inoculation. Bacteria are dividing and are free in the intercellular space (i). Resistant and hypersensitive reactions appear to be similar. v=vacuole, c=chloroplast. ×9,790. **9,** The resistant combination (*P. syringae*/WBR 133 bean) as seen 4 hr after inoculation. Bacteria are multiplying in the intercellular space. Most cells and bacteria (b) appear to be normal. Susceptible and hypersensitive reactions appear to be similar. w=host cell wall. ×12,460. **10,** The hypersensitive combination (*P. coronafaciens*/Tenderwhite bean) as seen 8 hr after inoculation. Multiplying bacteria are occupying much of the intercellular space. There is no sign of bacterial envelopment. Host cells show signs of damage at this time (arrows). Susceptible and resistant reactions appear to be similar. c=chloroplast. ×7,120. **11,** The resistant combination (*P. syringae*/WBR 133 bean) as seen 8 hr after inoculation. One of the few examples of bacterial envelopment seen in leaves. Note separation of the plasmalemma (p) from the host cell wall and accumulation of vesicles between them (arrows). Most bacteria are free and appear to be normal. ×20,470.

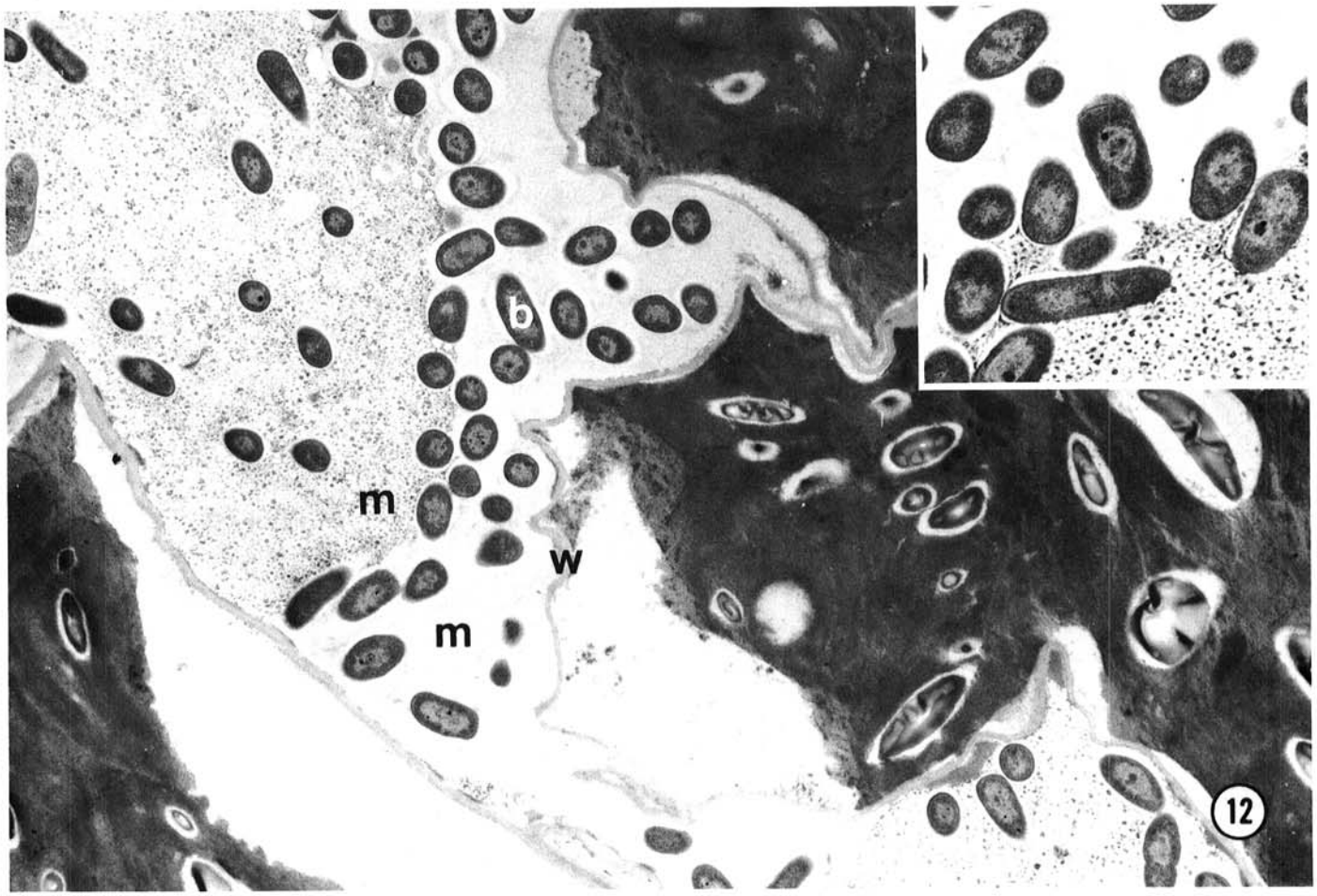


Fig. 12. The susceptible combination (*P. syringae*/ Tenderwhite bean) as seen 24 hr after inoculation. Host cells are completely collapsed. Bacteria (b) fill the intercellular spaces and appear normal. Note the matrix (m) surrounding the bacteria (insert). Resistant and hypersensitive reactions appear to be similar. w = host cell wall. × 8,800.

coronafaciens decreased with increasing inoculum doses, as has been reported by Ercolani and Crosse (8) for *P. phaseolicola* and *P. morsprunorum* inoculated into heterologous hosts (bean or cherry).

There are reports that the hypersensitive necrosis may not be related to the termination of pathogen growth. Király et al (11) found that the multiplication of *P. pisi* and *P. syringae* inoculated into tobacco did not decrease at the time of the appearance of the hypersensitive necrosis (7–9 hr), but rather, continued 24–72 hr before leveling off. Furthermore, multiplication of these pathogens was the same whether the hypersensitive necrosis was allowed to develop or was inhibited by infiltrating albumin into the intercellular spaces.

There were few differences at the ultrastructural level that could account for differences in symptom expression and in growth kinetics in the three host-bacteria combinations we studied. In leaves, signs of a morphologically induced defense response were seen only rarely and occurred in all three combinations. In all three combinations bacteria multiplied in the intercellular spaces and host cell collapse occurred at the same time. Alosi et al (1) suggested that the envelopment response seen in beans is nonspecific, and our data with leaf tissue would support this. But the initial stages of what appeared to be an envelopment response specific for bacteria in the resistant and hypersensitive combinations were seen in pods, although bacteria continued to multiply in the intercellular spaces. This was perhaps caused by the use of abnormally high doses of bacteria, resulting in almost identical multiplication patterns in the three combinations. Studies at low inoculum doses would be needed to substantiate this, but are not feasible at the electron microscope level. Growth curves are different in leaves and pods, and it is possible that the defense response also differs. Based on our data, however, we have no evidence that envelopment and immobilization factors are significant defenses of beans against these bacteria. Further studies will be needed to determine the nature of resistance.

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