Leaf Damage as a Predisposing Factor in the Infection of Apple Shoots by Erwinia amylovora

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Missouri Agricultural Experiment Station Journal Series Paper No. 7187.
Accepted for publication 30 August 1971.

ABSTRACT

Spraying uninjured apple shoots with suspensions of *Erwinia amylovora* had no effect, but extensive blight developed when the leaves were damaged before inoculation by excising the apices of the laminae. The incidence of blight decreased as the interval between injury and inoculation was increased, but residual susceptibility remained after 48 hr. There was evidence that the bacteria penetrated into leaves through the severed ends of the xylem vessels, then migrated in these elements to the shoot axis. The incidence of leaf and of shoot infection increased with inoculum concentration or inoculum dose (ID) in a typical dosage:response relationship. The median ID(50) for shoot infection was estimated to be 38 bacterial cells, but some shoots were probably infected from a single cell. Infection of the shoot axis most commonly originated through the first fully unrolled leaf and the next leaf below. These leaves were also the most susceptible to infection at the higher inoculum concentrations, but at lower concentrations the position of maximum susceptibility shifted to slightly older leaves. Reasons for this interaction between leaf age and inoculum concentration are discussed.

Additional key words: inoculum potential.

Phytopathology 62:176-182.

There are several references in the literature to fireblight infections of apple and pear shoots originating in leaves damaged by wind, hail, and other agencies. Apart from some early work by Brooks (2), who found that infections occurred most readily through veinal injuries, and from experiments with insect vectors by Plurad et al. (14), comparatively little seems to be known about the mechanisms of this type of infection. Our attention was drawn to this problem by recent outbreaks in English (E. Billings, *personal communication*) and Missouri apple orchards, in which extensive shoot blight was associated with widespread wind damage. Because of its possible bearing on the epidemiology and control of the disease, we have investigated further the relationship between leaf damage and shoot blight of apple.

MATERIALS AND METHODS.—The experiments were performed in the greenhouse, using 1-year-old trees of the susceptible cultivar, Jonathan, and a Missouri strain of *Erwinia amylovora*, E9. Inoculum was prepared from 24-hr bacterial growth on Difco nutrient agar + 2% glycerol slopes (28 C). Water was used to suspend bacteria in the first experiment, but was subsequently replaced by 0.05 M potassium phosphate buffer (pH 6.5) because of loss of viability in aqueous suspension at the lower inoculum concentrations. Suspensions were adjusted turbidimetrically, and the concentration of viable bacteria determined immediately before inoculation by plating on Difco nutrient agar containing 5% sucrose and 2 μg/ml crystal violet. Leaves were injured by puncturing the mesophyll with a needle, or by excising ca. 1 cm from the apices of the laminae. Inoculum was applied to shoots as a spray, or "injected" into the xylem of leaves by submerging the cut ends of the laminae in bacterial suspensions. The latter technique was similar to that described by Roach (17) for injecting nutrients and dyes into leaves. The first fully unrolled leaf (L0) and next 5 leaves below (L1-5 inclusive) were inoculated on each shoot, and treatments were replicated on from 5 to 14 shoots (30 to 84 leaves). The length of L0 at time of inoculation varied between 1.5 and 2.0 cm. Leaf infections and shoot blight; i.e., shoots killed by systemic infection of the axis, were recorded up to 14 days after inoculation. In experiments varying inoculum concentration and dose, the median-infective concentrations and doses (IC 50 and ID 50, respectively) were calculated by the moving averages method of Thompson (20). Material for histological examination was sectioned in paraffin and stained by Stoughton's (19) method.

RESULTS.—*Effect of leaf injury on infection of shoots.*—Extensive blight developed in shoots with the leaf apices excised immediately before inoculation, but none developed in the uninjured controls (Table 1). Shoots were sufficiently susceptible after injury for an infection to occur with the inoculum concentration 10^3 cells/ml. The rate of symptom development, however, was markedly accelerated at the higher inoculum concentrations. The next experiment compared the effects of excising leaf apices with the effects of injuries resulting from multiple needle punctures (20/leaf) in the interveinal regions of the mesophyll. Shoots were sprayed with inoculum at an intermediate concentration ($10^{5.2}$ cells/ml) immediately after injury, and again there was no infection of the uninjured controls. After 7 days, however, ca. 7 of 16 shoots with excised leaves were showing systemic blight symptoms, and the remainder localized lesions in the axis of the shoot. Small necrotic areas appeared at the sites of the

TABLE 1. Effect of inoculum concentration on infection of apple shoots spray-inoculated on leaves with excised apices

Inoculum[a] concentration (cells/ml)	No. blighted shoots after 14 days	
	Apices excised	Uninjured controls
10^7	6/6	0/7
$<10^5$	4/6	0/7
$<10^3$	1/7	0/7
Check (H_2O)	0/7	0/7

[a] Bacteria in distilled water sprayed on shoots.

needle punctures, but there was no systemic infection, and only two shoots developed axial lesions. Significantly, both these arose from leaves where the veinal reticulum was opened by slight tearing of the laminae which occurred during puncturing.

Development of symptoms.—These followed the same pattern in all experiments, irrespective of treatment or method of inoculation. The first signs of infection appeared in the leaves after 2-4 days in the form of a linear necrosis extending down the main vein for varying distances from the cut edge of the leaf. This symptom was frequently followed by wilting and necrosis of the adjacent mesophyll, with the formation of large sectorial necroses at the distal end of the leaves resembling those sometimes observed in field infections. Occasionally, necrosis was restricted to a small patch of tissue adjacent to the main vein at some distance from the severed edge of the leaf. The next stage in symptom development was the appearance, after 5-8 days, of localized lesions in the axis of the shoot, subtending infected leaves. In a few cases, the disease progressed no further and the affected shoots continued apparently normal growth. Systemic infection of shoots usually became evident between 6 and 12 days as a recurving of the shoot tip. The shoots subsequently turned brown and withered. The younger leaves were the most susceptible to infection and, in our experiments, most frequently gave rise to systemic shoot infection. In a typical experiment, inoculating the youngest fully unrolled leaf resulted in systemic infection of 61% of the shoots. When the second and third youngest leaves were inoculated, systemic infections were produced in 47 and 11% of the shoots, respectively. Inoculating older leaves than these failed to cause systemic infections.

Effect of inoculum concentration and dose on leaf and shoot infections.—Leaves were injected by immersing them for 5 sec in bacterial suspensions adjusted to give a logarithmic series of concentrations in the range $10^{6 \cdot 4}$-$10^{2 \cdot 4}$ cells/ml, inclusive. When the percentage incidences of shoot and of leaf infections were plotted against the log inoculum concentration, sigmoid dosage response curves were obtained. For shoot blight, the IC 50 was estimated at $10^{4 \cdot 2}$; and for leaf infections, at $10^{4 \cdot 4}$ cells/ml. Although infection increased with inoculum concentration, there was no indication in this experiment of the numbers of bacteria entering the leaves during

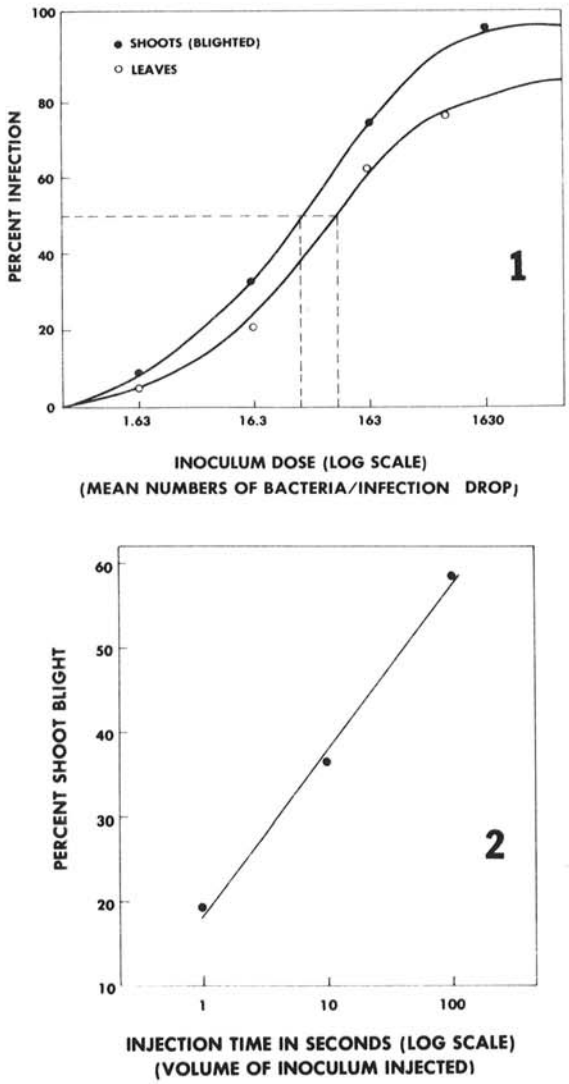

Fig. 1-2. Effects of inoculum dose and time of injection (inoculum volume) on the incidence of shoot and leaf infections of apple caused by *Erwinia amylovora*. **1)** Leaves inoculated with drops of equal volume deposited on the severed ends of main veins. **2)** Inoculum injected into leaves by submerging cut ends into bacterial suspensions.

infection; i.e., the inoculum dose. To determine the relationship between infection and dose, we injected leaves with small drops containing measured numbers of bacteria. A graded series of inocula in the range 10^5 -10^2 cells/ml were prepared, and drops of mean volume 6 μliters deposited from each concentration onto the cut ends of the main leaf veins with a microsyringe. Drops of phosphate buffer were used for the controls. The mean number of bacteria per drop at 10^3 cells/ml was found from plating five drops separately to be 16.3 ($\pm$ 2.1). Numbers at other concentrations were computed from this. Except on the youngest leaves, inoculum containing drops disappeared within 5 min. This was due to intake by the leaf, as drops deposited on undamaged petioles

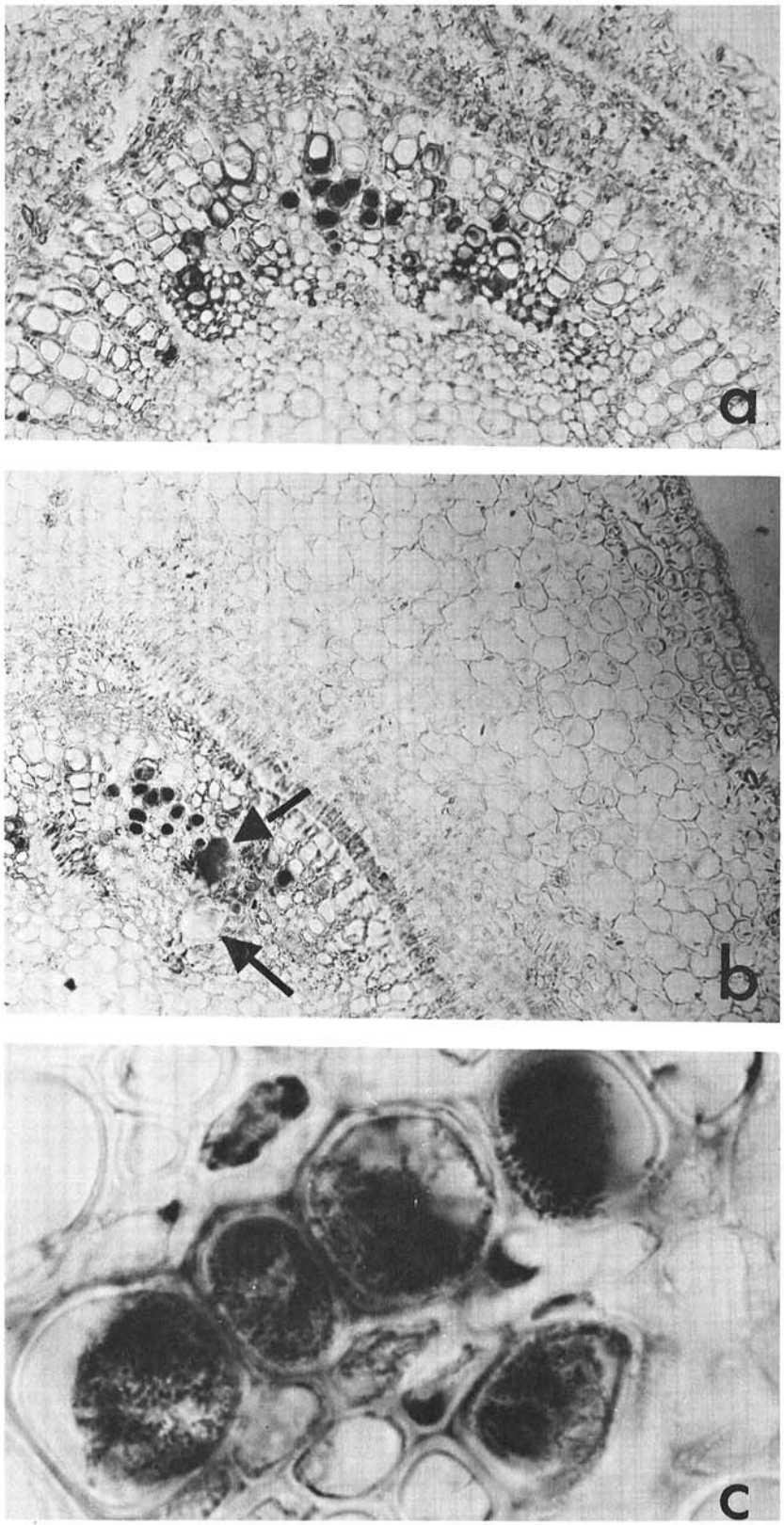

Fig. 3. Apparently healthy petioles in which *Erwinia amylovora* migrated from leaf laminae to shoot axes. **a, c)** Bacteria confined entirely to the xylem vessels of the leaf traces. **b)** Beginnings of migration into the medulla with the formation of lysigenous cavities (arrowed).

and laminae persisted unchanged during this period. On the youngest leaves, the drops persisted longer and the effective volume was frequently reduced by overspill onto the laminae surfaces. No infection occurred in the noninoculated controls. In the inoculation treatments, the relationship between log dose and infection was also sigmoid (Fig. 1), with ID 50's for shoot and leaf infections calculated at 38 and 102 bacteria, respectively. The minimum number of bacteria required for infection was obviously extremely low. Extrapolation from the response curve suggested that infection in some instances may have arisen from a single bacterium.

Penetration and migration of the pathogen.—When the cut edge of a leaf is immersed in a liquid, the liquid is immediately sucked into the xylem vessels by the negative tension in the water-conducting system (17). The process is continuous, and the volume of liquid entering the vessels is proportional to the length of time the leaves are immersed (injection time). Vascular suction has been shown to be the mechanism responsible for the penetration of pathogens through leaf scars in diseases of olive (9), cherry (3, 4), and apple (6). The probability that *E. amylovora* entered apple leaves in the same way was confirmed by microscopic examination of leaves after injecting them with India ink or suspensions of stained bacteria, using methods previously described (3). By far the greatest numbers of bacteria or India ink particles present in the leaves after injection were observed in the vessels of the main veins.

The question next arose whether these intratrachaeal bacteria were the cause of systemic shoot infections. If so, it followed that increasing the volume of inoculum (and hence the number of bacteria) injected into the vessels would have the same effect on blight incidence as would increasing the inoculum concentration. To test this we injected leaves for 1, 10, or 100 sec with inoculum at a fixed concentration near the IC 50 level determined in the previous experiment. The resulting incidence of blight per cent was approximately linearly related to log time; i.e., to log inoculum volume (Fig. 2). Since this line corresponds to the central part of the log concentration or log dose/response curves (Fig. 1), it is clear that the effects of inoculum concentration and inoculum volume were the same. The incidence of shoot blight in our experiments was therefore directly related to, and probably determined by, the numbers of bacteria entering the vessels of the leaves.

The passage of bacteria in vessels during injection is impeded by internal thickening of the walls, by cross-walls, and by anastamoses in the vascular system (4). The effect of this continual "sieving" process is to restrict initial penetration by removing bacteria from suspension. We have no direct evidence on the depth of initial penetration by *E. amylovora*, except from the experiment with stained suspensions, where bacteria were observed in vessels up to 3 mm below the cut edge of the leaf. It is certain, however, that some bacteria penetrated much deeper than indicated by this technique, which was capable of detecting bacteria only in mass. The depth of penetration is probably reflected by the length of the necrosis appearing in the main veins after 2-4 days. This varied from ca. 1-30 mm, and as might be expected from a process influenced by a random sieving effect, it tended to increase with the numbers of bacteria injected into the vessels.

The fate of bacteria after lodgement in the vessels is not known, but they may undergo a short period of multiplication in situ before beginning active migration to the shoot axis. There was evidence that this migration took place also in the vessels. It was frequently noted that when lesions first appeared in the shoot axis, the petioles of the leaves from which they originated were still green and turgid. In sections taken from petioles at this stage, bacteria were mainly confined to the vessels, particularly to the older centripetal vessels of the central vascular traces (Fig. 3). In a very few instances, the bacteria had escaped from the vessels to form small lysigenous pockets in the adjacent medullary parenchyma, but there was no sign, in any of the material examined, of bacteria in the cortex or phloem. These observations in no way conflict with Lewis & Goodman's (12) evidence that migration following hydathode or trichome infection of leaves is primarily in the phloem. On the contrary, they indicate that more than one migratory pathway is open to the pathogen, and that the pathway used may depend on the original mode of ingress.

Effect of delaying inoculation of leaves after injury.—In diseases originating through leaf scars, the incidence of infection decreased progressively as the interval between defoliation and inoculation was lengthened (3, 6, 9). A similar phenomenon was observed by Brooks (2) in apple shoots infected with *E. amylovora* through wounds. To examine the effects of delaying inoculation in our system, we injected leaves for 5 sec with $10^{5.2}$ cells/ml at intervals of 0, 24, and 48 hr after excision of the apices. The results confirmed that blight occurred most readily through fresh wounds, although there was residual susceptibility up to 48 hr (Table 2). The reduction in blight after 24 hr was related to a decrease in the length of veinal necrosis, and after 48 hr to a decrease in the number of leaf infections. This suggests that blight was first limited by the depth of penetration of inoculum, but at a later stage by the number of leaves in which the number of bacteria penetrating was above the threshold for infection. This interpretation is consistent with results described from experiments on the penetration of India ink and dye solutions through leaf scars (3, 6, 9).

Relationship between leaf susceptibility and leaf age.—The susceptibility of leaves to infection tended to decrease with age, but was affected also by the inoculum concentration and inoculum dose (Fig. 4). Thus at $10^{6.4}$ and $10^{5.4}$ cells/ml L0, the youngest leaf was most susceptible, and susceptibility then declined progressively with leaf age. At $10^{4.4}$ cells/ml, the position of maximum susceptibility shifted to L2, and at $10^{3.4}$ cells/ml, to L3. Similar interactions were observed with inoculum dose (Fig. 4). Calculation of the IC 50's for different leaves

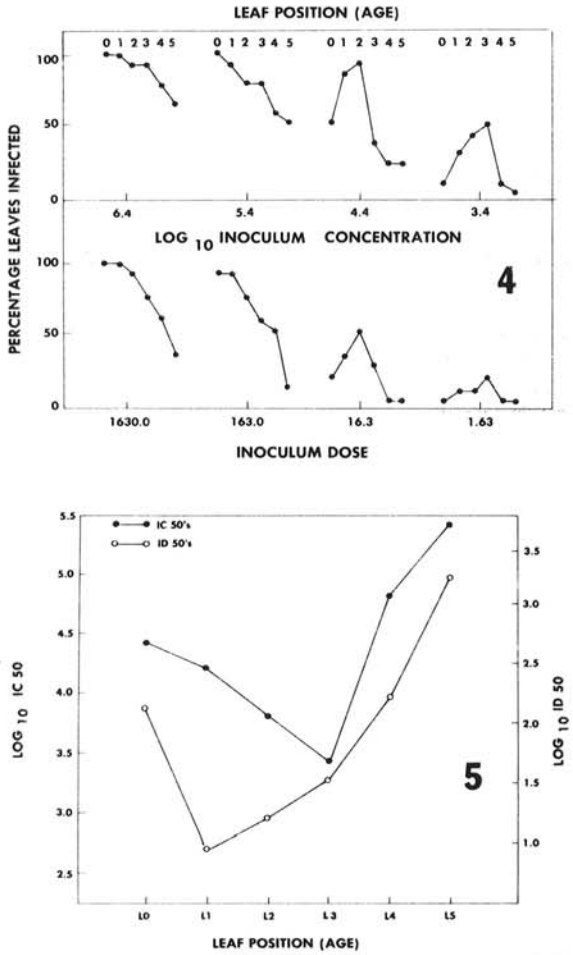

Fig. 4-5. Relationship between leaf age and susceptibility of leaves to infection with *Erwinia amylovora.* **4)** Effect of inoculum concentration and inoculum dose on the infection of leaves at different ages (L0-L5, inclusive). **5)** Median infective inoculum concentrations (IC 50) and median inoculum doses (ID 50) for leaves of different ages. The figures shown on the graph are the arithmetic values of the ID 50's. L0 = the first fully unrolled leaf, and L15 = the next 5 leaves below.

showed that these decreased between L0 and L3 inclusive, then increased. The ID 50's followed a different pattern, increasing progressively with leaf age except between L0 and L1 (Fig. 5). These results suggest that different factors were limiting infection of younger and of older leaves.

The infection of younger leaves was probably limited by the rate of intake of inoculum during injection. We have already noted that when drops were deposited on the cut ends of the main veins, these disappeared more slowly into younger leaves. An examination of the intensity and length of visible staining in the main veins of leaves injected by immersion for a standard time in 0.1% alcoholic crystal violet or 1.0% aqueous acid fuchsin confirmed that intake of liquids into younger leaves was slower. These observations suggest that in the experiment varying inoculum concentration, less inoculum entered the younger leaves during the standard 5 sec the leaves were immersed in the suspensions. This was not critical for the infection of younger leaves when the inoculum concentration was high, but evidently became so when the concentration was $10^{4 \cdot 4}$ cells/ml or less (Fig. 4).

In the experiment varying inoculum dose, the volume of inoculum was determined by the size of the drops placed on the main veins, and was independent of the rate of uptake. Although the drops were of uniform size, some deposited on younger leaves were lost by overspill onto the laminae, thus reducing the mean volume of inoculum injected in these leaves. We believe this accounts for at least part of the interactions observed between leaf age and the inoculum dose.

The main factor limiting infection of older leaves was undoubtedly the increasing numbers of bacteria required to infect leaves as they matured. This is reflected in the increase in ID 50's, from 9 to 1,620, between leaves L1 and L5, inclusive (see Fig. 5).

DISCUSSION.–Under the conditions of our experiments, leaf injury was essential for the induction of shoot blight. Age of leaf, the nature of the injury, and the inoculum dose were also important. Our results showed that leaves are liable to infection at any age, but the younger are most susceptible and most likely to give rise to systemic infections of the shoot axis. As leaves mature, increasing numbers of bacteria are required to infect them. Two possible explanations for this increase are as follows. The first assumes that leaf infections originate from single bacteria, but that each bacterium has only a limited chance after inoculation of reaching a site in the vessels suitable for

TABLE 2. Effect on shoot and leaf infection[a] of delaying inoculation after wounding

	Interval between wounding[b] and inoculation (hr)		
	0	24	48
Shoots blighted (%)	71	33	9
Leaves infected (%)	63	79	28
Mean length of veinal necrosis[c] (mm)	21	12	12

[a] Extent of infection was noted 8 days after inoculation.
[b] Apical excisions.
[c] Infected leaves only.

multiplication. The probability of infection, therefore, depends on the number of bacteria inoculated and the number of sites available. If fewer sites are available in older leaves, because of structural and other modifications during maturation, then greater numbers of bacteria would be required to induce infection at the same level of probability as in younger leaves. The alternative explanation postulates qualitative changes in the infection sites of older leaves, such that they are no longer competent to support the growth of a single bacterium; bacteria must now cooperate during infection in order to "saturate" host defenses. Increasing numbers of bacteria are required to infect leaves as they age, because of the increasing effectiveness of the leaf defensive systems. These two explanations are based on the hypotheses of "independent action" and of "cooperative action", respectively. These hypotheses seek to explain why, in most infectivity titrations, a given host responds consistently to inoculation only when challenged with several bacteria. They are discussed in detail by Meynell & Stocker (15), and their application to a plant disease is described by Ercolani (7, 8).

Leaf injuries must be sufficiently extensive to involve the veinal reticulum, and so provide the pathogen with direct access into the vascular system of the leaf. This agrees with the results of earlier work described by Brooks (2). Bacteria are sucked through the broken ends of the xylem vessels, and migrate in these elements to the shoot axis. The infectivity of *E. amylovora* by this route is extremely high, and infection can probably arise from the penetration of a single bacterium. A similar order of infectivity has been observed in blossom infections. Thus, Hildebrand (10) obtained 60% infection of apple blossoms with single cell inocula, and a comparable level of infection was reported by Ivanoff & Keitt (11) in pear blossoms inoculated with doses of ca. 10 cells.

When all other factors for infection are equal, the size of the inoculum dose is decisive. Under field conditions, dosage will be determined in the first instance by the concentration of bacteria in infection drops at the site of injury. We have detected large numbers of *E. amylovora* on the leaves of field apple trees shortly after the appearance of blight symptoms, but there seems to be no comparable information on the magnitude of leaf surface populations prior to infection, or on the extent to which these decay with time. On greenhouse trees we observed (5) an asymptotic decrease in epiphytic populations of *E. amylovora* from ca. 10^8 to 10^6/leaf in 11 days, equivalent to a survival rate over this period of 0.1%; the survival rate after 24 hr was 10%; after 48 hr, 1%. Assuming a similar decay on field trees, it is clear that maximum populations will be expected on leaves immediately after the dispersal of inoculum from the primary sources. Since leaves are most susceptible when freshly injured, this suggests optimal conditions for infection when injury and dispersal occur simultaneously. These conditions are likely to be fulfilled in epidemics originating in wind or windblown rain. The size of the inoculum dose in field infection will also depend on the volume of inoculum entering the leaves. This process requires free water, and therefore the duration of leaf wetness after arrival of the inoculum may be important when the rate of injection is low. The rate of injection is related to the tension in the water-conducting system, and will tend to be low when soil moisture is high and transpiratory activity low. Under these conditions, cherry leaf scars were found to be less susceptible to infection by *Pseudomonas morsprunorum* (4).

Although leaf injury was a major predisposing factor in our experiments, there is no reason to believe that it is essential for field infection. On the contrary, we have observed field infections of apple shoots in the absence of any visible injury, and several workers have succeeded inducing blight by inoculating leaves through natural openings (1, 12, (16, 18, 21). Bauske (1) concluded that the state of leaf maturity was more important than injury in the development of pear epiphytotics. Rosen (18), on the other hand, considered mechanical wounds to be an important factor in field infection. Pierstoff (13) and Brooks (2), who observed negligible infection from the inoculation of uninjured leaves, shared this opinion.

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