Protection of Pear Against Fire Blight by Bacteria and Bacterial Sonicates

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ABSTRACT

A delay in the expression of fire blight symptoms occurred in actively growing 'Bartlett' pear shoots when inoculation with 1.2×10^6 cells of avirulent *Erwinia amylovora*, *E. herbicola*, or *Pseudomonas tabaci* (inducers) preceded inoculation with the same concentration of virulent *E. amylovora* cells (challenge) by 24 hr. In some experiments this delay appeared permanent. A delay in symptom expression did not occur when the challenge followed the inducers by 0.5 hr or when *Xanthomonas campestris* was used as the inducer. Similar results were obtained in 10-day-old etiolated Bartlett pear seedlings. A delay in symptom expression occurred when inoculation with 10^6 cells of the inducers preceded inoculation with 10^4, 10^3, or 10^2 cells of the challenge by 24 hr. In several experiments, delay in symptom expression occurred when challenge followed inducer by 0.5 hr. No delay was noted when *X. campestris* was used as the inducer. The similarities in response between clonal Bartlett pear shoots and etiolated seedlings indicate that etiolated seedlings may be used to study the nature of the protection. A delay in symptom expression also occurred when cell-free sonicates of both avirulent and virulent *E. amylovora* were used as inducers. In vitro experiments showed that sonicates did not inhibit the growth or virulence of *E. amylovora*.

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It has been observed that prior inoculation of the host plant with avirulent mutants of pathogens (2, 18), nonpathogens (5), auxotrophic mutants of nonpathogens (5), and epiphytic bacteria normally found on the host surface (3) protect against bacterial pathogens. Similar responses have also been noted with disrupted cells of avirulent mutants (2), cell-free extracts of pathogens and nonpathogens (23), heat-killed cells of pathogens (15, 23), nonpathogens (23), and avirulent mutants (2).

In 1928, Rosen (22) inferred that an unrelated yellow schizomycete limited the progress of *Erwinia amylovora* since it persisted in cankers after the pathogen could no longer be isolated. Farabee & Lockwood (6) later reported that a nonpathogenic yellow bacterium isolated from fire blight cankers inhibited growth of *E. amylovora* in vitro and in vivo. Goodman (7, 8) made further observations that the yellow bacterium, now often referred to as *E. herbicola* (4), avirulent *E. amylovora*, and *Pseudomonas tabaci* protected 'Jonathan' apple from fire blight. A similar response on clonal 'Bartlett' pear and etiolated Bartlett pear seedlings has been reported by McIntyre & Williams (19). Wrather (24) noted protection against fire blight with these same inducers in fruits of mature 'Anjou' and immature Bartlett pear, and immature Jonathan and 'Red Delicious' apple.

MATERIALS AND METHODS.—Actively growing clonal *Pyrus communis* L. 'Bartlett' seedlings at least 30 cm in height were used in all experiments involving the use of green tissues. Plants were grown individually in clay pots containing soil and maintained in a plastic greenhouse at ca. 25 C and a relative humidity greater than 90%. Etiolated Bartlett open-pollinated seedlings were grown in a mixture of vermiculite and soil (6:4) for 10 days at 19 C in the dark. Seedlings 4.5- to 5.5-cm tall were gently removed from the soil, washed several times with tap water to remove soil particles, and rinsed with distilled water. These plants were maintained on moist filter paper between two pyrex baking dishes covered with aluminum foil.

Virulent *E. amylovora* (challenge) and *E. herbicola* (inducer) were isolated from lyophilized, naturally infected apple buds, and a change in our virulent isolate during culturing yielded an avirulent *E. amylovora*. These bacteria were grown on modified Emerson's Agar (21) for 24 hr. *Pseudomonas tabaci* (obtained from R. N. Goodman, University of Missouri) and *Xanthomonas campestris* (obtained from J. Tuite, Purdue University) were also used as inducers and were grown for the same time period on nutrient agar and potato-dextrose agar, respectively. Bacteria were suspended in 0.05 M phosphate buffer at pH 6.5 (9), and the concentration of inoculum was adjusted spectrophotometrically.

Pear shoots were inoculated with a bacterial suspension by the use of a syringe and 25-gauge, one-half-inch needle. All inoculations (inducer and challenge) were made ca. 2.0 cm below the shoot apex by passing the needle through the stem and leaving a drop of inoculum on the wound as the needle was withdrawn. The drop, containing ca. 1.2×10^6 cells, was quickly drawn into the plant through the hole left by the needle. Either 0.5 or 24 hr after the inducer was injected, the challenge was applied in the same manner and at the same concentration.

TABLE 1. Protection of 'Bartlett' pear shoots against fire blight by avirulent *Erwinia amylovora*, *E. herbicola*, *Pseudomonas tabaci*, or *Xanthomonas campestris*[a]

Treatment and time of inoculation (hr)			Shoots with symptoms and days after inoculation				
0	0.5	24	4	6	8	10	t Value[b]
Buffer	Buffer		0	0	0	0	
Buffer	Inducers[c]		0	0	0	0	
Buffer	V[d]		5	5	5	5	
Buffer		V	5	5	5	5	
AV	V		5	5	5	5	
AV		V	1	1	2	5	11.67**
Y	V		5	5	5	5	
Y		V	1	2	2	2	13.25**
PT	V		1	3	3	5	2.50
PT		V	1	1	1	2	15.20**
XC	V		4	5	5	5	1.00
XC		V	2	4	5	5	1.43

[a] Five plants per treatment; inoculum concentration = 1.2×10^6 cells.

[b] t Value calculated with paired t-test. Values significant at 0.01 (**) level.

[c] Inducers: AV = avirulent *E. amylovora*; Y = *E. herbicola*; PT = *P. tabaci*; XC = *X. campestris*.

[d] Challenge: V = virulent *E. amylovora*.

Etiolated pear seedlings were inoculated with 10 μliters of a bacterial suspension injected ca. 1.0 cm below the cotyledons. Both inducer and challenge were inoculated at the same point on the hypocotyl. Because of the extreme susceptibility of etiolated seedlings, the concentration of inducers was reduced to 1.0×10^6 cells, and that of challenge was reduced to 10^4, 10^3, or 10^2 cells, with challenge following inducer by either 0.5 or 24 hr.

Cell-free sonicates of virulent and avirulent *E. amylovora* were prepared by suspending ca. 1.0 g of cells in phosphate buffer and centrifuging at 20,000 *g* for 20 min. The pellet was resuspended in 15 ml phosphate buffer and sonicated on ice for eight 30-sec intervals, and the sonicate was centrifuged as before. The supernatant was filtered twice through a 0.22-μ Millipore membrane and checked for sterility by streaking the filtered sonicate on plates of modified Emerson's Agar. The sterile cell-free sonicates were adjusted to contain ca. 1,000 μg/ml protein by the Lowry method. Ten-day-old etiolated Bartlett pear seedlings were injected as previously described with 10 μliters of sonicate followed 0.5 or 24 hr later with 10^3 cells of challenge, a concentration found to give excellent results in both control and treated seedlings.

To determine whether cell-free sonicates of virulent or avirulent *E. amylovora* affected the growth of virulent *E. amylovora* in vitro, the sonicates were prepared as before, and 7.0-ml aliquots

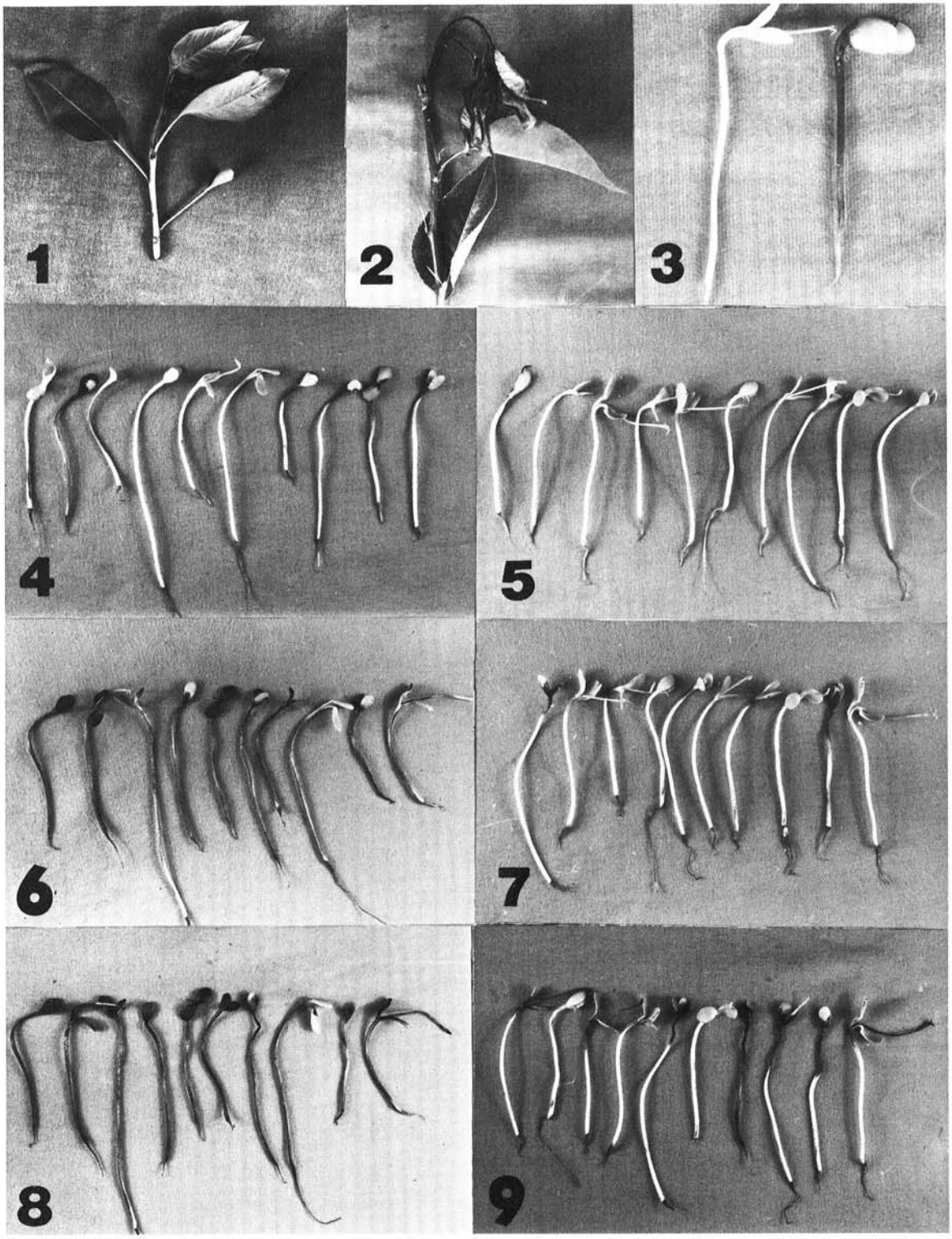

Fig. 1-9. 1) Protected and **2)** Control clonal 'Bartlett' pear 6 days after challenge with 10^6 cells of *Erwinia amylovora*. The symptomless plant was inoculated with 10^6 cells of inducers (avirulent *E. amylovora, E. herbicola*, or *Pseudomonas tabaci*) 24 hr before challenge. **3-9)** Etiolated Bartlett pear seedlings. **3)** Protected and control 4 days after challenge with 10^3 cells *E. amylovora*. The symptomless seedling was inoculated with 10^6 cells inducers (avirulent *E. amylovora, E. herbicola*, or *P. tabaci*) 24 hr before challenge. **4, 6, 8)** Control 2, 4, and 6 days after challenge with 10^3 cells of *E. amylovora*. **5, 7, 9)** Treated with sonicate of avirulent *E. amylovora* 24 hr before challenge with 10^3 cells *E. amylovora* 2, 4, and 6 days after challenge.

of sonicate were placed in 250-ml flasks with 100 ml of a mineral medium containing 1% sucrose (1). The flasks were then inoculated with 2.0×10^3 cells of virulent *E. amylovora*. Controls consisted of 7.0 ml phosphate buffer in place of the sonicates, and growth of the bacterium was observed spectrophotometrically at 525 nm. All experiments were repeated a minimum of three times, and the data are presented as averages of the total number of observations.

RESULTS.—Avirulent *E. amylovora, E. herbicola*, and *P. tabaci* caused a delay in the expression of fire blight symptoms in clonal Bartlett pear shoots when challenge followed these inducers by 24 hr (Table 1, Fig. 1, 2). This delay was not evident when challenge followed these inducers by 0.5 hr (Table 1). Symptom expression was not delayed when *X. campestris* was used as the inducer. Similar results were obtained when these inducers were used in etiolated seedlings (Table 2, Fig. 3). Avirulent *E. amylovora, E. herbicola*, and *P. tabaci* appeared to delay the expression of fire blight symptoms when challenge followed the inducers by 0.5 hr, but in only three cases was this delay statistically significant (Table 2).

Cell-free sonicates of both virulent and avirulent *E. amylovora* were found to delay fire blight symptoms when challenge followed injection of the sonicates by 24 hr (Table 3, Fig. 4-9), but not when challenge was injected 0.5 hr after the sonicates. In vitro experiments showed that the medium containing sonicates supported growth of *E. amylovora* (Table 4). Within 3 days after inoculation, sonicates increased growth 1,600 to 2,600% over controls. Typical fire blight symptoms occurred when bacteria grown under these conditions for 3 days were injected into etiolated pear seedlings.

DISCUSSION.—Goodman (8) demonstrated that avirulent *E. amylovora, E. herbicola*, and *P. tabaci* protected Jonathan apple from fire blight. We have shown that these inducers delay the expression of fire blight symptoms in clonal pear shoots when the challenge followed the inducers by 24 hr but not when the challenge followed the inducers by 0.5 hr. Wrather (24) noted a similar occurrence in immature Bartlett pear fruit with the same inducers. Goodman

TABLE 2. Protection of etiolated 'Bartlett' pear seedlings against fire blight by avirulent *Erwinia amylovora, E. herbicola, Pseudomonas tabaci*, or *Xanthomonas campestris*[a]

Treatment and time of inoculation (hr)			Seedlings with symptoms and days after inoculation					
0	0.5	24	2	3	4	5	6	t Value[b]
Buffer	Buffer		0	0	0	0	0	
Buffer	Inducers[c]		0	0	0	0	0	
Buffer	V^4 or 3^d		10	10	10	10	10	
Buffer		V^4 or 3	10	10	10	10	10	
Buffer	V^2[d]		8	10	10	10	10	
Buffer		V^2	6	10	10	10	10	
AV	V^4		4	10	10	10	10	1.00
AV		V^4	4	6	6	6	10	3.67*
AV	V^3		2	4	8	10	10	1.95
AV		V^3	2	2	3	6	6	6.77**
AV	V^2		0	8	8	8	10	2.06
AV		V^2	0	0	0	0	2	11.00**
Y	V^4		0	10	10	10	10	1.00
Y		V^4	2	3	6	7	8	4.15*
Y	V^3		0	7	9	9	10	1.45
Y		V^3	0	1	6	7	7	3.79*
Y	V^2		0	1	4	8	8	3.68*
Y		V^2	0	3	6	6	6	7.91**
PT	V^4		0	2	4	6	8	4.24*
PT		V^4	4	6	6	8	8	4.81**
PT	V^3		0	0	6	8	10	2.53
PT		V^3	3	4	4	4	4	31.00**
PT	V^2		0	4	6	8	8	3.77*
PT		V^2	1	2	2	2	2	12.33**
XC	V^4		8	10	10	10	10	2.00
XC		V^4	8	10	10	10	10	2.00
XC	V^3		10	10	10	10	10	
XC		V^3	10	10	10	10	10	
XC	V^2		8	8	10	10	10	2.00
XC		V^2	6	8	10	10	10	2.00

[a] Ten plants per treatment.
[b] t Value calculated with paired t-test. Values significant at 0.05 (*) or 0.01 (**) level.
[c] Inducers (10^6 cells): AV = avirulent *E. amylovora*; Y = *E. herbicola*; PT = *P. tabaci*; XC = *X. campestris*.
[d] Challenge: V^4, V^3, V^2 = 10^4, 10^3, 10^2 cells of virulent *E. amylovora*, respectively.

TABLE 3. Protection of etiolated 'Bartlett' pear seedlings against fire blight by cell-free sonicates of virulent or avirulent *Erwinia amylovora*[a]

Treatment and time of inoculation (hr)			Seedlings with symptoms and days after inoculation					
0	0.5	24	2	3	4	5	6	t Value[b]
Buffer	Buffer		0	0	0	0	0	
Buffer	Sonicates[c]		0	0	0	0	0	
Buffer	V[d]		13	18	20	20	20	
Buffer		V	14	18	20	20	20	
Son-Av	V		15	19	20	20	20	1.25
Son-Av		V	2	7	12	13	16	5.87**
Son-V	V		16	19	20	20	20	1.33
Son-V		V	2	10	14	16	18	3.72*

[a] Twenty plants per treatment; ca. 1,000 μg/ml protein in the sonicates.
[b] t Value calculated with paired t-test. Values significant at 0.05 (*) or 0.01 (**) level.
[c] Sonicates: Son-Av = sonicate of avirulent *E. amylovora*; Son-V = sonicate of virulent *E. amylovora*.
[d] Challenge: V = 10^3 cells of virulent *E. amylovora*.

(8) reported protection of Jonathan apple when the challenge followed *E. herbicola* by 0.5 or 24 hr. We inoculated a number of clonal Jonathan apple seedlings in the same manner as previously described for pear to determine whether this protection, when challenge followed inducer by 0.5 hr, was due to differences in our experimental methods or differences in the host. A significant delay in symptom expression occurred when inducer preceded challenge by both 0.5 and 24 hr, suggesting that there are inherent differences between Bartlett pear and Jonathan apple in their relationship to *E. amylovora* and/or these inducers.

Keen & Horsch (14) warned against the use of "unnatural" host-parasite systems. In both clonal pear shoots and etiolated seedlings there is a delay in symptom expression when challenge follows the inducers by 24 hr, and no protection was noted in either system when *X. campestris* was the inducer (Table 1, 2). This suggests that the two systems are comparable, and that the use of etiolated seedlings should be beneficial in the study of protection against fire blight since they make available large quantities of uniform, highly susceptible, tissue which can be maintained under controlled conditions.

Protection of etiolated pear seedlings with cell-free sonicates of avirulent and virulent *E. amylovora* (Table 3), and the observation that these sonicates do not inhibit reproduction (Table 4) or affect the virulence of *E. amylovora* in vitro, suggest that induced resistance does occur in Bartlett pear. Since sonicates and living inducer gave similar results (Table 2, 3), it appears that host response is a major factor in resistance rather than competition between inducer and challenge.

TABLE 4. Growth of *Erwinia amylovora* in the presence of sonicates of virulent or avirulent *E. amylovora*[a]

Time after inoculation (hr)	Number of bacterial cells/ml		
	Control	Son-Av	Son-V
0	16	16	16
24	5.0×10^5	2.1×10^7	2.0×10^7
48	1.5×10^7	6.0×10^8	2.5×10^8
72	3.0×10^7	8.0×10^8	5.0×10^8

[a] Control: 100 ml mineral medium containing 1% sucrose, 7.0-ml buffer, and inoculated with 2.0×10^3 cells virulent *E. amylovora*. Test: as control with 7.0 ml sonicate of virulent (Son-V) or avirulent (Son-Av) *E. amylovora* in place of the buffer. Sonicates contained ca. 1,000 μg/ml protein.

Results of other studies (15, 23) on bacteria have indicated that induced protection is temporary; these results are in accord with most of ours. Protection appeared to be permanent in several clonal pears, but this was not the general case. Several experiments with etiolated seedlings have also given results which indicated that some plants may be permanently protected. However, the plants are difficult to maintain for more than 14 days, which eliminates the opportunity for prolonged observation. Nevertheless, permanent protection with some bacteria has been observed (2).

The nature of the factor(s) responsible for protection in our studies is unknown. Production of a phytoalexin-like inhibitory substance may be involved, as has been hypothesized by others working with bacterial systems (2, 15).

Hildebrand & Schroth (12) found that hydroquinone, the aglycon of arbutin, inhibited the growth of *E. amylovora*. They later reported (13) that the antibiotic activity induced in leaf discs was dependent upon β-glucosidase levels rather than the amounts of arbutin present in the tissue. Although further reports (11, 20) were contradictory as to the importance of β-glucosidase for resistance of pear to fire blight, Hildebrand (10) reported that β-glucosidase is involved in the degradation of arbutin to liberate antibacterial quantities of hydroquinone. These reports suggest that, as in tobacco where induced resistance to wildfire disease is associated with increased peroxidase activity (16, 17), induced resistance to fire blight in Bartlett pear is associated with changes in the levels of arbutin and/or β-glucosidase.

LITERATURE CITED

1. ARK, P. A. 1937. Variability in the fire-blight organism, Erwinia amylovora. Phytopathology 27:1-28.
2. CARROLL, R. B., & F. L. LUKEZIC. 1972. Induced resistance in alfalfa to Corynebacterium insidiosum by prior treatment with avirulent cells. Phytopathology 62:555-564.
3. CROSSE, J. E. 1965. Bacterial canker of stone-fruits. VI. Inhibition of leaf-scar infection of cherry by a saprophytic bacterium from the leaf surface. Ann. Appl. Biol. 56:144-160.
4. DYE, D. W. 1969. A taxonomic study of the genus Erwinia. III. The herbicola group. N. Z. J. Sci. 12:223-236.
5. ERCOLANI, G. L. 1970. Bacterial wilt of tomato. IV. The interaction between virulent and avirulent strains of Corynebacterium michiganense (E. F. Sm.) Jens. in vivo. Phytopathol. Mediterr. 9:151-159.
6. FARABEE, G. J., & J. L. LOCKWOOD. 1958. Inhibition of Erwinia amylovora by bacterium species isolated from fireblight cankers. Phytopathology 48:209-211.
7. GOODMAN, R. N. 1965. In vitro and in vivo interactions between components of mixed bacterial cultures isolated from apple buds. Phytopathology 55:217-221.
8. GOODMAN, R. N. 1967. The protection of apple stem tissue against Erwinia amylovora infection by avirulent strains and three other bacterial species. Phytopathology 57:22-24.
9. GOODMAN, R. N., & W. H. SHAFFER. 1971. An inoculation procedure for evaluating the efficacy of toxicants against Erwinia amylovora. Proc. Second Workshop Fire Blight Res., Michigan State Univ.
10. HILDEBRAND, D. C. 1970. Fire blight resistance in Pyrus: hydroquinone formation as related to antibiotic activity. Can. J. Bot. 48:177-181.
11. HILDEBRAND, D. C., C. C. POWELL, JR., & M. N. SCHROTH. 1969. Fire blight resistance in Pyrus: Localization of arbutin and β-glucosidase. Phytopathology 59:1534-1539.
12. HILDEBRAND, D. C., & M. N. SCHROTH. 1963. Relation of arbutin-hydroquinone in pear blossoms to invasion by E. amylovora. Nature 197:513.
13. HILDEBRAND, D. C., & M. N. SCHROTH. 1964. Antibiotic activity of pear leaves against Erwinia amylovora and its relation to β-glucosidase. Phytopathology 54:59-63.
14. KEEN, N. T., & R. HORSCH. 1972. Hydroxyphaseollin production by various soybean tissues: a warning against use of "unnatural" host-parasite systems. Phytopathology 62:439-442.
15. LOVREKOVICH, L., & G. L. FARKAS. 1965. Induced protection against wildfire disease in tobacco leaves treated with heat-killed bacteria. Nature 205:823-824.
16. LOVREKOVICH, L., H. LOVREKOVICH, & M. A. STAHMANN. 1968. The importance of peroxidase in the wildfire disease. Phytopathology 58:193-198.
17. LOVREKOVICH, L., H. LOVREKOVICH, & M. A. STAHMANN. 1968. Tobacco mosaic virus-induced resistance to Pseudomonas tabaci in tobacco. Phytopathology 58:1034-1035.
18. MAIN, C. E. 1968. Induced resistance to bacterial wilt in susceptible tobacco cuttings pretreated with avirulent mutants of Pseudomonas solanacearum. Phytopathology 58:1058-1059 (Abstr.).
19. MC INTYRE, J. L., & E. B. WILLIAMS. 1972. Protection of Bartlett pear by avirulent Erwinia spp. and Pseudomonas tabaci. Phytopathology 62:777 (Abstr.).
20. POWELL, C. C., JR., & D. C. HILDEBRAND. 1967. β-glucosidase content of pear blossom tissues and its relation to antibiotic activity of the tissues against Erwinia amylovora. Phytopathology 57:826 (Abstr.).
21. REINHARDT, J. F., & D. POWELL. 1960. Culture media for viability studies and storage of Erwinia amylovora. Phytopathology 50:685-686.
22. ROSEN, H. R. 1928. Variation within a bacterial species. I. Morphological variations. Mycologia 20:251-275.
23. SLEESMAN, H. C., J. E. PERLEY, & H. A. J. HOITINK. 1970. Susceptible and hypersensitive reactions in tobacco and their prevention by cell-free extracts of Pseudomonas tabaci and Pseudomonas glycinea. Phytopathology 60:1314 (Abstr.).
24. WRATHER, J. A. 1972. Inhibition of fire blight in pome fruits by non-pathogenic bacteria and effect of light on susceptibility of etiolated seedlings. M.S. Thesis, Purdue Univ. 64 p.