Induced Resistance in Alfalfa to Corynebacterium insidiosum by Prior Treatment With Avirulent Cells

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ABSTRACT

Resistance to virulent isolates of *Corynebacterium insidiosum* was induced in four susceptible alfalfa cultivars (DuPuits, Cardinal, Sonora, Moapa) by leaflet infiltration with suspensions of avirulent mutants from the same original single-celled culture. Similar tests on small root sections of greenhouse and gnotobiotically grown alfalfa, maintained in a moist chamber, gave the same results as those obtained with infiltrated leaves. Challenge tests showed that partial protection occurred as early as 6 hr, and protection was maximum or near-maximum by 12 hr. Challenge tests on companion leaflets indicated that the protection factor(s) is not translocated or light-dependent. Protection in leaflets and root sections was also induced by suspensions of avirulent cells that had been Formalin-treated, sonicated, or sonicated and then filtered through a .45-μ Millipore membrane. Protection was not elicited by cell-free filtrates of nonsonicated avirulent cells or by suspensions of virulent cells treated in the same manner as the avirulent cells. Protection was not induced in root sections by effective or noneffective isolates of *Rhizobium meliloti*, or by four isolates of unidentified bacteria obtained from alfalfa roots. Protection was obtained when roots of seedlings and mature plants grown under gnotobiotic conditions were preinoculated with avirulent *C. insidiosum* cells.

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Virus and fungus infections often protect plant tissues against subsequent infection with the same or another pathogen. This phenomenon of an immunelike response in plants is not uncommon (24), and was first demonstrated for viruses by Kunkel (11) in 1934. More recently, Loebenstein (12) demonstrated that the protective effect of some plant viruses is not necessarily due to the initially administered virus itself, but rather to a substance(s) elaborated by the host in response to initial infection. Similar protective phenomena have been demonstrated recently for bacterial diseases of plants. Lovrekovich & Farkas (13) showed that pretreatment of tobacco leaves by heat-killed cells of *Pseudomonas tabaci* induced protection when the leaves were subsequently challenge-inoculated with live virulent cells of the same pathogen. Main (17) pretreated susceptible tobacco cuttings with avirulent mutants of *Pseudomonas solanacearum*, and observed protection when they were subsequently challenged with virulent bacteria. Similarly, Goodman (6) reported that apple shoots inoculated with avirulent isolates of *Erwinia amylovora*, a yellow *Erwinia*-like isolate, and *P. tabaci* were protected against infection when subsequently inoculated with a virulent strain of *E. amylovora*. Ercolani (4) showed that tomato plants preinoculated with either *P. fluorescens* or an auxotrophic mutant of *P. syringae* were protected against infection by *Corynebacterium michiganense*. He also showed that heat-killed cells of *P. syringae* and *C. michiganense* induced less protection than live cells.

Viral-induced protection in plants against pathogenic bacteria was demonstrated by Lovrekovich et al. (15). They reported that tobacco leaves infected with tobacco mosaic virus developed resistance to wildfire caused by *P. tabaci*. The induced resistance was not localized to leaf tissue immediately surrounding the lesions, but also affected host tissue relatively distant from the local lesions.

The primary purpose of the study described herein was to determine whether the phenomenon of induced resistance could be stimulated in alfalfa (*Medicago sativa* L.) against *Corynebacterium insidiosum* (McCull.) H. L. Jens. by prior treatment with whole cells and preparations from avirulent cells of *C. insidiosum*, and by other bacteria.

MATERIALS AND METHODS.—*Preparation of inoculum.*—Avirulent and virulent isolates of *C. insidiosum* utilized in these studies were originally derived from single cells and maintained by the methods previously described (1).

Inoculum was prepared by gently washing the bacteria from a 7- to 10-day-old culture, on beef lactose agar (BLA), with sterile distilled water. The inoculum was standardized to a concentration of ca. 2.2×10^8 cells/ml with a spectrophotometer. In preliminary tests, this concentration of inoculum was observed to give consistently good results and was used throughout these studies. The bacteria were always washed from the culture surface and dilutions were made just before using them.

Challenge tests on root sections.—Investigations to

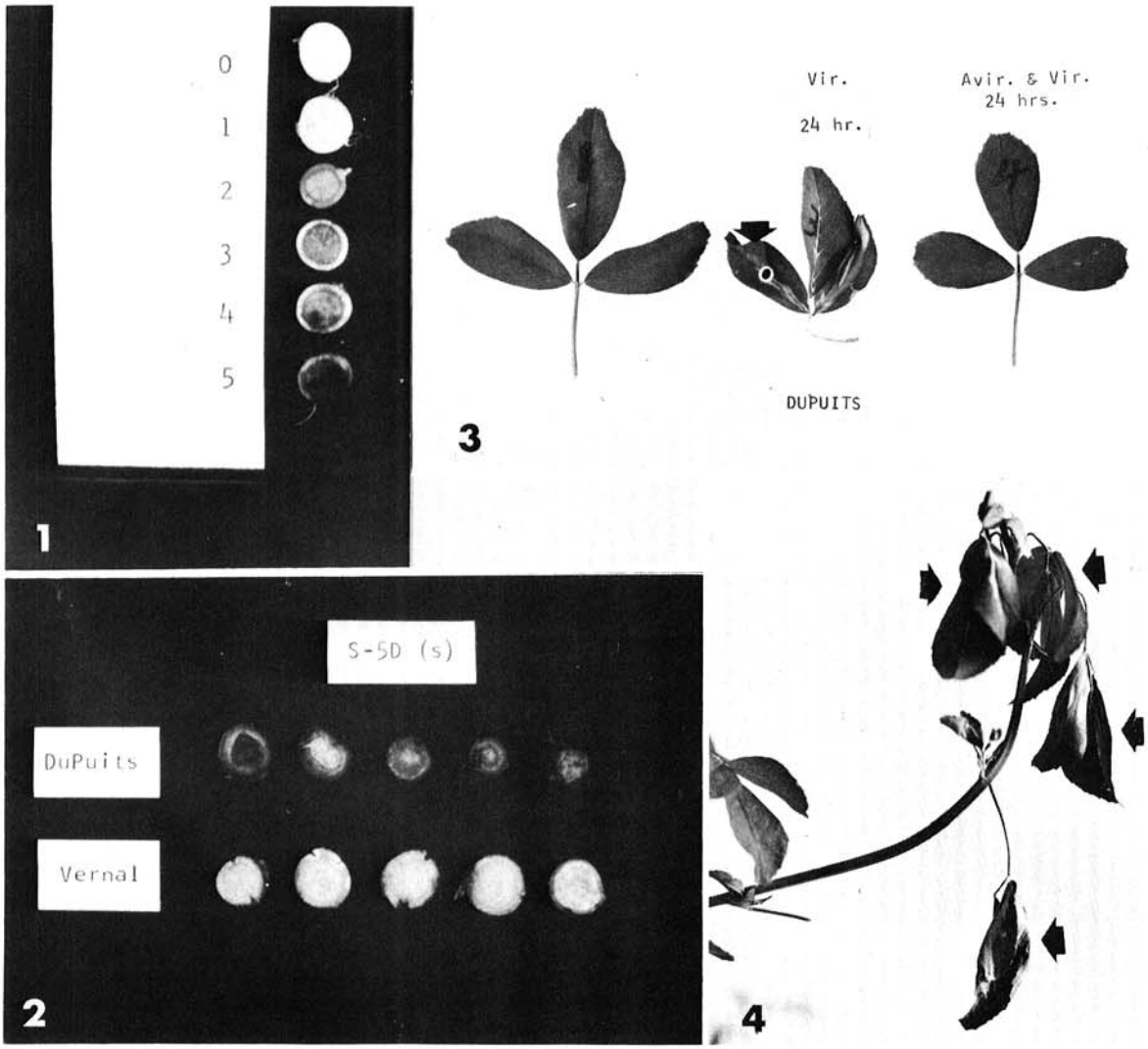

Fig. 1-4. 1) Scale used for rating discoloration of alfalfa root sections. 0 = no apparent discoloration; 1 = a few brownish spots in the vascular cylinder; 2 = slight brownish streaks appearing in the stele; 3 = extensive streaks appearing in the stele; 4 = entire stele dark brown and cortex becoming discolored; 5 = entire section dark brown to black. **2)** Reaction of section of resistant (Vernal) and susceptible (DuPuits) roots of gnotobiotically grown alfalfa to a virulent isolate (S-5D) of *Corynebacterium insidiosum.* **3)** Protection induced in DuPuits alfalfa leaflets by prior infiltration with avirulent cells of *Corynebacterium insidiosum* (S-5D). The control, infiltrated with water, is on the left. Note the necrotic tissue (indicated by the arrows) in the unprotected trifoliolate. **4)** Reaction of DuPuits alfalfa leaflets to infiltration with virulent cells of *Corynebacterium insidiosum* (S-5D) 72 hr after inoculation. (Arrows indicate collapsed trifoliolates).

determine whether a resistant reaction could be induced in roots, and to study the conditions influencing it, were completed using roots of mature alfalfa plants (four resistant and four susceptible cultivars) grown in the greenhouse. Clean roots that were not discolored were cut into ca. 5-cm sections and surface-sterilized in 70% ethanol for 2 min, followed by 3 min in a 10% solution of Clorox (5.25% sodium hypochlorite by wt). These were rinsed 6 times (2 min/rinse) in sterile distilled water, cut into small sections (ca. 0.4 cm thick and 0.7 cm diam), and placed on end in small petri dishes containing filter paper moistened with sterile Hoagland's solution No. 1 (9) supplemented with 0.1% glucose.

The freshly cut root sections were inoculated by placing a 5-μliter drop of the appropriate bacterial suspension on the upper surface with a microsyringe. Controls were treated with the same amount of sterile water. Inoculated sections of the resistant cultivar, Vernal, were also included as controls in each experiment. All steps were completed under aseptic conditions. Inoculated sections were incubated in the dark at 21 C and evaluated at the first sign of any

discoloration in the controls (usually 3-5 days). Sections were rated according to the following scale (illustrated in Fig. 1): 0 = no apparent discoloration; 1 = a few brownish spots in the vascular cylinder; 2 = slight brownish streaks appearing in the stele; 3 = extensive streaks appearing in the stele; 4 = entire stele dark brown and cortex becoming discolored; 5 = entire section dark brown to black.

Alteration of host response to virulent isolates of *C. insidiosum* was determined by prior inoculation with five avirulent isolates of *C. insidiosum*, with three gram-negative (A-1, B-1, C-1) and one gram-positive (N-1) bacterial isolates from alfalfa roots, and with two isolates, one effective (Rh-21) and one noneffective (Rh-26), of *Rhizobium meliloti*. In each case, the test bacterium was inoculated alone on the root sections and challenge-inoculated at 12, 24, and 36 hr with virulent bacteria. Challenge inoculations were also made at 12 and 24 hr after inoculation with suspensions of avirulent and virulent cells derived from the green or pink colony types of *C. insidiosum*, respectively, described in a previous study (2).

To determine if killed or otherwise treated *C. insidiosum* cells could induce protection in root sections, avirulent and virulent cells were treated as follows: (i) killed by heating at 37 C for 12 hr in a 1% Formalin solution (5) (Formalin was removed, under sterile conditions, by alternate washing and centrifugation); (ii) killed by sonification (in the cold) for 20 min at a setting of 7 on a Model IS - 75 Branson Sonifier; (iii) filtration through a .45-μ Millipore membrane; and (iv) sonification prior to filtration. Formalin-treated and filtered bacterial suspensions were checked for the presence of live cells by a streaking of the suspensions onto BLA plates which were incubated at 21 C.

Mature Vernal and DuPuits roots produced under gnotobiotic conditions (16) were used to prove that only the test organism, and not other microorganisms naturally present in root sections, was responsible for the induced resistance. That such roots were microbe-free was thoroughly checked by culturing subsamples at 21 and 32 C on potato-dextrose agar, beef lactose agar, nutrient agar, Czapek-Dox agar, nutrient broth, and liquid thioglycollate medium.

Challenge tests by infiltration of leaflets.—To study whether protection could be induced in mature alfalfa leaves, leaflets of intact plants were infiltrated with bacterial suspensions using the device described by Hagborg (7). This device was modified to provide a chamber large enough to encompass most of the leaflet. Since preliminary tests indicated that ca. 0.25 ml of the bacterial suspensions was satisfactory both for infiltration and for obtaining results, this amount was used throughout these studies.

Five avirulent-virulent combinations of *C. insidiosum* isolates were tested on four resistant (Vernal, Buffalo, Saranac, and Lahontan) and four susceptible (DuPuits, Moapa, Sonora, and Cardinal) alfalfa cultivars. Five leaflets/cultivar (one/trifoliolate) were infiltrated for each treatment, which were as follows: (i) avirulent cells alone; (ii) virulent cells alone; (iii) avirulent cells then challenge inoculated after 12, 24, and 36 hr with virulent cells; and (iv) controls infiltrated with an equal amount of sterile distilled water at the various time intervals. Control leaflets were on the same trifoliolate leaves as the other treatments.

We ascertained the effect of light on the protection phenomenon by infiltrating leaflets with the avirulent isolates of the bacterium and covering them with aluminum foil until challenged 24 hr later. In another series, leaflets were covered for 24 hr after the challenge inoculation. The same cultivars included in the previous experiment were tested, but only two isolates of *C. insidiosum* were included.

To determine if the protective factor(s) is translocatable, one leaflet in a trifoliolate was inoculated with avirulent cells, and the opposing leaflet, on the same trifoliolate, was challenged 24 hr later with virulent cells. Controls were the same as previously described.

The same treatments described for the root section tests were used to determine if killed and variously treated *C. insidiosum* cells could induce protection in alfalfa leaves. Only one isolate was used in these tests conducted on DuPuits and Vernal leaves.

The following scale was used to evaluate the results of all leaflet infiltration tests: 0 = no reaction; 1 = slight chlorosis; 2 = definite yellowing; 3 = small amount of necrosis (less than one-third of leaflet area); 4 = large amount of necrosis (one-third to two-thirds of leaflet area); 5 = entire leaflet necrotic; and 6 = necrosis extending into petiole.

Challenge tests on seedlings.—Four resistant (Vernal, Buffalo, Saranac, and Lahontan) and four susceptible (DuPuits, Moapa, Narragansett, and Cardinal) cultivars of alfalfa were grown under sterile conditions in large test tubes by the method previously described (1). We inoculated the seedlings, when 6 weeks old, by wounding the roots with a long, fine needle and dispensing 2 ml of inoculum into the sand. Four avirulent-virulent combinations of *C. insidiosum* isolates were included in the tests. Treatments consisted of: (i) avirulent cells only; (ii) virulent cells only at 0 hr; (iii) virulent cells only at 24 hr; (iv) avirulent cells followed by challenge with virulent cells after 24 hr; and (v) controls injured but treated with sterile distilled water instead of inoculum. All treatments consisted of 10 seedlings/cultivar (a total of 80 seedlings) except that, for iv, 30 seedlings/cultivar (total of 120 seedlings) were used. Also, 10 seedlings of each of the resistant cultivars were inoculated with each of the virulent isolates. Disease ratings using a scale similar to that of Cormack et al. (3) were made 4 weeks after inoculation.

Challenge tests on mature plants.—Mature resistant (Vernal) and susceptible (DuPuits) alfalfa plants grown in an isolator providing a gnotobiotic environment (16) were inoculated by wounding the roots with a long, fine needle, then pouring the inoculum (10 ml/container) into the sand surrounding the roots. These plants were clipped to a

TABLE 1. Reaction of root sections of alfalfa cultivars to treatment with avirulent cells of *Corynebacterium insidiosum*

Cv	Isolate	Avirulent, 0 hr	Virulent		Avirulent + Virulent, 24 hr	H_2O Control
			0 hr	24 hr		
Resistant						
Buffalo	S-5D	0.6[a,b]	0.9	0.7	0.9	0.5
Saranac	S-5D	0.7	0.4	0.6	0.3	0.6
Lahontan	S-5D	0.4	0.9	0.2	0.5	0.7
Vernal	S-5D	0.3	0.2	0.1	0.2	0.4
Vernal	S-5B	0.2	0.5	0.4	0.3	0.3
Vernal	G-1F	0.9	0.4	0.7	0.4	0.5
Susceptible						
Moapa	S-5D	0.9	2.5	2.1	0.1	0.6
Sonora	S-5D	0.5	3.4	3.3	1.4	0.7
Cardinal	S-5D	1.1	3.6	3.4	1.0	1.0
DuPuits	S-5D	0.5	2.2	2.5	0.2	0.3
DuPuits	S-5B	1.0	3.4	3.6	0.9	0.5
DuPuits	G-1F	0.9	2.9	2.9	1.1	0.8

[a]Average of 10 sections/treatment.
[b]0 = no apparent discoloration; 5 = entire section brown to black (see Fig. 1).

TABLE 2. Effect of pretreatment with different bacteria on induction of protection against *Corynebacterium insidiosum* in alfalfa root sections

Isolate	Source	DuPuits			Vernal
		Isolate alone	Challenged, 24 hr	Virulent[a]	Isolate alone
A-1	Wilt-infected DuPuits alfalfa roots	2.3[b,c]	4.4	4.5	2.3
B-1	Buffalo alfalfa roots	1.6	3.8	4.5	4.1
C-1	Cherokee alfalfa roots	1.8	4.3	4.5	2.4
N-1	Niagara alfalfa roots	2.3	4.4	4.5	2.3
Rh-21	Effective *Rhizobium meliloti* from alfalfa roots	1.4	4.4	4.5	2.5
Rh-26	Noneffective *Rhizobium meliloti* from alfalfa roots	2.9	4.5	4.5	

[a]Virulent *C. insidiosum* cells, isolate S-5D.
[b]Average of 20 sections/treatment.
[c]0 = no apparent discoloration; 5 = entire section brown to black (see Fig. 1).

uniform height (6 cm) and watered prior to the application of inoculum. The treatments were as follows: (i) inoculation with avirulent cells only; (ii) inoculation with virulent cells only; (iii) inoculation with avirulent cells and subsequent challenge inoculation after 24 hr with the virulent cells; and (iv) 16 control plants injured and treated with water. The treated plants were separated as far as possible in the isolator to avoid any cross-contamination of bacteria. Plants were harvested and rated for disease development according to a scale similar to that of Cormack et al. (3).

RESULTS.—*Challenge tests on root sections.*—The interaction of root sections of four resistant and susceptible alfalfa cultivars and avirulent and virulent cells of *C. insidiosum* is shown in Table 1. High discoloration ratings were obtained for all the susceptible cultivars inoculated with virulent cells alone, but not for the resistant ones (Fig. 1). Protection was observed in all the susceptible cultivars treated first with avirulent cells, then challenged with virulent cells. This was not due to a change in virulence of the virulent inoculum as shown by the readings made at 0 and 24 hr. Controls gave reaction ratings of 0.3-1.0. Similar results were obtained when sections were pretreated with avirulent cells of the green colony type prior to challenge with virulent cells of the pink colony type.

Evidence that the protection response observed in root sections was induced by avirulent cells of *C. insidiosum* and not by other bacteria naturally present in roots was obtained with roots grown gnotobiotically and determined by extensive testing to be microbe-free. Induction of protection against *C. insidiosum* in root sections apparently is specific for avirulent cells of the bacterium (Table 2). The four unidentified bacteria from alfalfa roots all failed to induce protection against *C. insidiosum* in root sections. The same was true for both the effective and the noneffective isolate of *Rhizobium meliloti*. Vernal root sections showed as much or more response to these bacteria than did DuPuits sections.

Induction of the protective response in root sections was effected by 12 hr, and remained constant over the 36-hr interval tested (Table 3). Tests on isolates S-2C and S-5D indicated that

TABLE 3. The influence of length of exposure to avirulent cells on induction of protection against *Corynebacterium insidiosum* in DuPuits alfalfa root sections

Isolate	Treatment	Time of treatment (hr) 0	6	12	24	36
G-1G	Avirulent	0.5[a,b]				
	Virulent	3.5		3.6	3.6	4.0
	Avirulent + Virulent			0.9	0.8	0.8
	Control	0.8				
G-1H	Avirulent	0.5				
	Virulent	2.6		2.3	2.8	2.8
	Avirulent + Virulent			0.9	0.6	0.4
	Control	0.6				
S-2C	Avirulent	0.6				
	Virulent	4.1	4.0	4.0	4.0	4.1
	Avirulent + Virulent		2.2	0.9	0.7	0.9
	Control	0.2				
S-2D	Avirulent	0.7				
	Virulent	4.1		3.9	3.6	4.0
	Avirulent + Virulent			0.8	0.8	0.6
	Control	0.4				
S-5D	Avirulent	0.8				
	Virulent	4.5	4.3	4.6	4.5	4.3
	Avirulent + Virulent		2.0	0.7	0.7	0.8
	Control	0.6				

[a]Average of 20 sections/treatment.

[b]0 = no apparent discoloration; 5 = entire section brown to black (see Fig. 1).

TABLE 4. Effect of treatment of *Corynebacterium insidiosum* cells (isolate S-5D) on induction of protection in DuPuits root sections and infiltrated leaflets

Treatment[a]	Rating[b] Roots	Leaves
Avirulent–0 hr	0.7[c]	0.9[d]
Virulent–0 hr	3.2	5.2
Virulent–24 hr	3.1	5.1
Avirulent + Virulent	1.2	1.1
H_2O–control	0.5	0.5
Avirulent (Formalin treated) + Virulent	0.8	1.0
Virulent (Formalin treated) + Virulent	3.0	4.6
Avirulent (Millipore filtered) + Virulent	4.2	5.1
Virulent (Millipore filtered) + Virulent	4.1	4.8
Avirulent (Sonicated) + Virulent	1.2	1.4
Virulent (Sonicated) + Virulent	3.2	4.5
Avirulent (Sonicated, then filtered) + Virulent	1.8	1.1
Virulent (Sonicated, then filtered) + Virulent	3.7	4.9

[a]All challenge inoculations made after 24 hr with virulent *C. insidiosum* cells.

[b]Average of 10 sections and 10 leaflets/treatment.

[c]0 = no apparent discoloration; 5 = entire section dark brown to black (see Fig. 1).

[d]0 = no reaction 6 = necrosis extending into petiole (see text).

induction had begun by 6 hr, but that the protective response was not maximum until 12 hr.

The effect of various treatments of *C. insidiosum* on the protection phenomenon is shown in Table 4. Avirulent cells that had been Formalin-treated, sonicated, or sonicated prior to Millipore filtration, were all capable of inducing protection in root sections. Cell-free filtrates of avirulent bacterial cultures obtained by Millipore filtration did not induce protection. Virulent cells, subjected to the same treatments, failed to induce protection in all instances.

Corynebacterium insidiosum could readily be recovered from inoculated root sections when isolations from these were attempted on BLA. However, some other bacteria also were observed infrequently in these isolations. Fungal contamination was rarely observed in plates containing the root sections. No attempt was made to determine the pathogenicity of the colonies of *C. insidiosum* or other microorganisms observed.

Challenge tests by infiltration of leaflets.—Table 5 and Fig. 3 show that protection against virulent *C. insidiosum* in alfalfa leaves can be induced by prior infiltration with avirulent cells of *C. insidiosum*. A high degree of protection against both isolates tested was induced in all four susceptible cultivars. The response of resistant cultivars to the virulent bacteria was minimal in comparison to that for susceptible cultivars. The response in the susceptible leaflets often appeared to be a hypersensitive one which consisted of very rapid necrosis and collapse of the tissue (Fig. 4). In extreme cases, the necrosis

TABLE 5. Reaction of infiltrated leaflets of alfalfa cultivars to treatment with avirulent and virulent cells of *Corynebacterium insidiosum*

Cv	Isolate	Avirulent	Virulent, 24 hr	Avirulent + Virulent, 24 hr	H_2O Control
Resistant					
Buffalo	S-5D	0.6[a,b]	0.9	0.8	0.2
	S-2C	0.2	0.7	0.5	0.2
Saranac	S-5D	0.3	1.3	1.0	0.2
	S-2C	0.3	1.4	0.9	0.4
Lahontan	S-5D	0.2	1.6	1.2	0.4
	S-2C	0.5	1.3	1.0	0.4
Vernal	S-5D	0.4	0.8	0.9	0.2
	S-2C	0.3	0.6	0.8	0.1
Susceptible					
Moapa	S-5D	0.2	5.1	0.3	0.1
	S-2C	0.5	5.3	0.4	0.4
Sonora	S-5D	0.3	5.5	0.3	0.2
	S-2C	0.4	5.4	0.2	0.3
Cardinal	S-5D	0.1	5.2	0.3	0.2
	S-2C	0.2	5.3	0.5	0.1
DuPuits	S-5D	0.3	5.6	0.3	0.4
	S-2C	0.2	5.4	0.7	0.3

[a]Average of 10 leaflets/treatment.

[b]0 = no reaction; 6 = necrosis extending into petiole (see text).

extended into the petiole. The reaction for the controls was minimal.

Inoculations made at different time intervals, following infiltration with avirulent cells, indicated that protection is induced by 12 hr and is essentially unchanged through 36 hr (Table 6). Control leaflets infiltrated at each of the time intervals gave no reaction, and virulence of the bacterium remained constant. This indicates that protection was not due to any sudden change(s) in the host or bacterium per se, but rather to an interaction of the two.

Light had no effect on induction of protection. Protection occurred against *C. insidiosum* in all four cultivars regardless of whether the infiltrated leaflets were covered prior to or after the challenge inoculation.

Evidence that the factor(s) responsible for induction of protection in alfalfa leaves is not translocated from one leaflet to another was obtained (Table 7). In no case was a leaflet protected, when subsequently challenged, because of prior inoculation of its companion leaflet with avirulent cells. This relationship held for all combinations of the four cultivars (DuPuits, Cardinal, Sonora, and Moapa) and two isolates (S-2C and S-5D) tested.

TABLE 6. The influence of length of exposure to avirulent cells on induction of protection against *Corynebacterium insidiosum* in infiltrated DuPuits alfalfa leaflets

Isolate		Time of treatment (hr) 0	12	24	36
G-1G	Avirulent	0.3[a,b]			
	Virulent	6.0	6.0	6.0	6.0
	Avirulent + Virulent		1.0	0.3	0.5
	Control[c]	0.0	0.0	0.0	0.0
G-1H	Avirulent	0.3			
	Virulent	4.3	4.5	4.5	4.0
	Avirulent + Virulent		1.3	0.8	0.8
	Control	0.3	0.0	0.3	0.3
S-2C	Avirulent	0.3			
	Virulent	4.3	4.5	4.0	4.3
	Avirulent + Virulent		0.0	0.0	0.8
	Control	0.0	0.0	0.5	0.0
S-2D	Avirulent	1.0			
	Virulent	3.3	3.0	3.5	3.3
	Avirulent + Virulent		0.5	0.8	0.0
	Control	0.3	0.0	0.0	0.0
S-5D	Avirulent	0.0			
	Virulent	5.3	5.0	6.0	5.3
	Avirulent + Virulent		1.3	0.5	0.0
	Control	0.0	0.0	0.5	0.5

[a]Average of four leaflets/treatment.

[b]0 = no reaction; 6 = necrosis extending into petiole (see text).

[c]Infiltrated with an equal amount of sterile distilled water.

The results of various treatments of *C. insidiosum* cells on their capacity to induce protection in infiltrated leaflets were essentially the same as those obtained with root sections (Table 4). Avirulent cells that were Formalin-treated, sonicated, or sonicated prior to Millipore filtration, all induced protection. Cell-free filtrates of the avirulent cultures obtained by Millipore filtration alone, along with all the treated virulent cells, failed to induce protection.

Isolations made from infiltrated leaflets, on BLA, showed that *C. insidiosum* could be recovered from inoculated leaves several days after infiltration. Other bacteria also were frequently isolated, particularly one that produced small, circular, yellow colonies.

Challenge tests on seedlings.—Protection was uniformly induced in four susceptible cultivars tested when treated with avirulent cells of *C. insidiosum* prior to inoculation with virulent cells (Table 8). All four isolates of the bacterium gave comparable results. All ratings for controls and resistant cultivars were extremely low. Also, there was no change in virulence of these bacteria whether inoculations were made at the same time or 24 hr after inoculation of the seedling with avirulent cells.

Random isolations made on BLA indicated that *C. insidiosum* could be recovered from inoculated seedlings of the susceptible cultivars regardless of treatment. However, the pathogen was more readily isolated from severely infected seedlings, and was not isolated from resistant cultivars in the few attempts made. Contaminants were not detected in these isolations, and controls were negative for any microbial growth.

Challenge test on mature plants.—The nature of the interaction between *C. insidiosum* and a resistant or susceptible alfalfa cultivar under gnotobiotic conditions is indicated in Table 9 and Fig. 5. Results of ratings made on a small number of mature plants, 5 weeks after inoculation, indicate that resistance was induced in the susceptible cultivar (DuPuits) by prior inoculation with avirulent cells of the bacterium (Fig. 6). This was further emphasized by comparison of regrowth, which was as good in the challenge-inoculated DuPuits plants as the control plants. The plants treated only with virulent bacteria showed wilting, small cupped leaves, bunchy-type growth, premature flowering, and typical discoloration of the taproot. Resistance remained stable in the normally resistant cultivar (Vernal).

Owing to the small number of plants tested, these results must be interpreted only as an indication that protection against *C. insidiosum* can also be elicited in mature alfalfa plants by prior treatment with avirulent cells.

DISCUSSION.—Results of this study indicate that protection against *C. insidiosum* can be induced in root sections and leaves of susceptible alfalfa cultivars by prior treatment with live, killed, or disrupted avirulent cells of the same bacterium. That induction of protection was obtained in seedlings and mature plants grown under gnotobiotic conditions is

TABLE 7. Results illustrating a lack of translocation of factor(s) responsible for induction of protection against *Corynebacterium insidiosum* in alfalfa trifoliolates

Isolate		Cv			
		DuPuits	Cardinal	Sonora	Moapa
S-2C	Avirulent–0 hr	0.3[a,b]	0.2	0.4	0.5
	Virulent–0 hr	5.4	5.3	5.4	5.1
	Avirulent + Virulent–24 hr	0.7	0.5	0.2	0.4
	Avirulent + Virulent on companion leaflet–24 hr	4.9	5.0	4.6	5.3
S-5D	Avirulent–0 hr	0.3	0.1	0.3	0.5
	Virulent–0 hr	5.6	5.2	5.5	5.1
	Avirulent + Virulent–24 hr	0.3	0.3	0.3	0.4
	Avirulent + Virulent on companion leaflet–24 hr	5.1	4.5	5.6	4.5

[a]Average of 10 leaflets/treatment.
[b]0 = no reaction; 6 = necrosis extending into petiole (see text).

TABLE 8. Disease development and induced protection against *Corynebacterium insidiosum* in four susceptible and four resistant cultivars of alfalfa seedlings grown under sterile conditions

Isolate[c]	Treatment	Susceptible cv[a]				Resistant cv[b]			
		1	2	3	4	1	2	3	4
G-1H	Avirulent	0.2[d,e]	0.8	0.4	0.2				
	Virulent–0 hr	4.4	4.2	4.4	4.4	1.0	0.7	0.7	0.9
	Virulent–24 hr	4.6	4.4	4.8	4.6				
	Avirulent + Virulent–24 hr	1.0	1.1	0.7	0.7				
	H_2O–Control	0.6	0.6	0.2	0.4	0.9	0.7	0.5	0.7
S-5D	Avirulent	0.8	0.6	0.8	0.6				
	Virulent–0 hr	4.2	4.6	4.0	4.2	0.4	0.8	0.9	0.8
	Virulent–24 hr	4.4	4.8	4.4	4.6				
	Avirulent + Virulent–24 hr	1.4	1.1	0.8	1.2				
	H_2O–Control	0.2	0.6	0.6	0.2	0.9	0.7	0.5	0.7

[a]1 = DuPuits; 2 = Moapa; 3 = Narragansett; 4 = Cardinal.
[b]1 = Vernal; 2 = Buffalo; 3 = Saranac; 4 = Lahontan.
[c]Only two isolates are shown as representative of the four tested.
[d]All values average of 10 seedlings/treatment except challenge treatment, which included 30 seedlings.
[e]0 = no infection; 5 = dead or dying (3). Evaluation made 4 weeks after inoculations.

especially significant, as under these conditions the induced protection is attributable solely to avirulent cells of *C. insidiosum*.

Our results are similar to those obtained in other studies. Mueller (18) found that sections of white potatoes treated with an avirulent strain of *Phytophthora* were resistant to a virulent strain. In contrast to Mueller's work, however, no necrotic reaction was associated with the induced resistance found in this study. Weber & Stahmann (21) also demonstrated that inoculation of sweet-potato root sections with a nonpathogenic isolate of *Ceratocystis fimbriata* induced, in a thin layer of tissue around the site of inoculation, an acquired immunity or resistance to subsequent inoculation with a pathogenic isolate. They did not find a necrotic reaction associated with the acquired immunity. In contrast to findings by Ercolani (4), who was able to induce resistance in tomato to *Corynebacterium michiganense* with bacteria other than avirulent isolates of *C. michiganense*, we were not able to obtain resistance with any bacteria tested except avirulent cultures of *C. insidiosum*.

The necrosis induced by leaflet infiltration and treatment of root sections occurred only with virulent cells in the compatible (susceptible) host. According to the definition of Klement & Goodman (10), this necrosis cannot be considered as involving a hypersensitive reaction (HR), since it did not occur in the incompatible host-pathogen combination. However, in infiltrated leaflets, the reaction was very rapid and involved collapse of host tissue that even in the most extreme cases did not extend beyond the petiole. Perhaps this reaction occurs because the bacterium is a wilt-inducing organism that normally infects the vascular system. All attempts to isolate the bacterium from stems and roots at different intervals after infiltration proved negative. Therefore, it would appear that the bacterium was confined to the necrotic area of the leaflet.

Previous work (6, 13) indicated that the protective effect of heat-killed bacteria is not

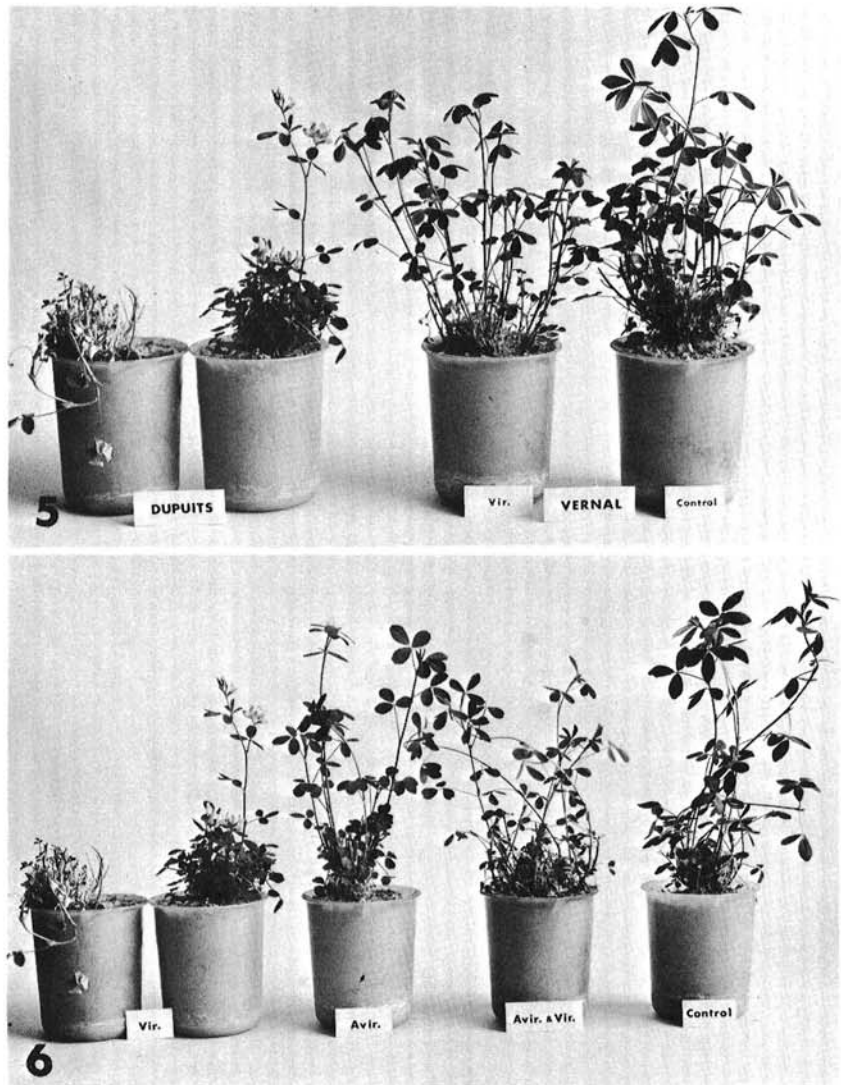

Fig. 5-6. 5) Reaction of DuPuits and Vernal alfalfa grown in a gnotobiotic environment to inoculation with virulent cells of *Corynebacterium insidiosum* (S-5D), 5 weeks after inoculation. **6)** Protection induced in DuPuits alfalfa grown in a gnotobiotic environment by prior inoculation with avirulent cells of *Corynebacterium insidiosum* (S-5D), 5 weeks after inoculation.

species-specific nor necessarily restricted to avirulent or closely related forms of the living pathogen. In the present study, the protective effect was not only specific for *C. insidiosum*, but also specific for avirulent cells of the bacterium whether living or killed. However, no attempt was made to test other pathogenic organisms. That dead cells could induce the reaction suggests the host tissue may play an active part in the process. Similarly, Weber & Stahmann (20) found that only nonpathogenic isolates of *Ceratocystis fimbriata* induced immunity to pathogenic isolates of the same fungus in sweet-potato roots.

Results of other studies (13, 18) on bacteria have indicated that induced protection is temporary, and only delays symptom expression for a few days. This was not true in the present study, as symptoms did not later appear in protected leaves.

Cell-free filtrates of avirulent cultures obtained by Millipore filtration alone failed to induce resistance. This is in contrast to the work of Lovrekovich et al. (14), who found that cell-free extracts of *Pseudomonas tabaci* could induce resistance in tobacco leaves. However, we found that when avirulent cells were sonicated prior to infiltration, protection was induced. Sleesman et al. (19) also found a fraction (heat labile) from sonicated cells of *Pseudomonas glycinea* that induced HR in infiltrated tobacco leaves.

We observed that induced protection was not

TABLE 9. The near absence of symptoms in inoculated Vernal alfalfa plants and induced protection against *Corynebacterium insidiosum* (S-5D) in mature DuPuits alfalfa plants grown in a gnotobiotic environment

Cv		Rating[a,b]	Regrowth (cm)[c]
DuPuits	Avirulent cells	0.7	19.7
	Virulent cells	4.0	8.6
	Avirulent + Virulent cells–24 hr	0.7	22.6
	H_2O–Control	0.5	21.4
Vernal	Avirulent cells	0.3	14.9
	Virulent cells	1.0	15.9
	Avirulent + Virulent cells–24 hr	0.6	21.0
	H_2O–Control	0.5	17.4

[a]Average of four plants/treatment except challenge treatment which included six plants.

[b]0 = no infection; 5 = dead or dying (3). Evaluation made 5 weeks after inoculations.

[c]Average of 16 stems/treatment except challenge treatment, which included 18 stems. Determined by measuring an equal number of the tallest stems per plant.

translocated from one leaflet to the companion leaflet on the same trifoliolate. Lovrekovich et al. (14) indicated that, in tobacco leaves "actively immunized" by infiltration with *P. tabaci*, resistance can be translocated to opposite halves from half-leaves treated with killed bacteria. We did not test this possibility.

The nature of the factor(s) responsible for the resistance determined in our study is unknown. Formation of phytoalexinlike inhibitory substances may be involved, as induction of phytoalexin in alfalfa leaves by certain fungi has recently been demonstrated (8).

Alterations in host proteins provide another possible explanation for the observed reaction and is being investigated. Lovrekovich et al. (14, 15) demonstrated increased peroxidase activity and induced formation of new isoenzymes of the enzyme in tobacco leaves injected with heat-killed cells of *P. tabaci* or infected with TMV. Both agents induced resistance which became apparent upon subsequent challenge of the leaves with virulent *P. tabaci* cells. Injection into the leaves of solutions of peroxidase also resulted in increased resistance. Considerable studies on induced immunity to *Ceratocystis fimbriata* in sweet-potato roots by prior treatment of tissues with nonpathogenic isolates have also indicated the important role played by alteration of host proteins, enzymes, and isoenzymes (22, 23, 24).

Further studies will be required to determine the nature of the factor(s) controlling the induced resistance observed in this study. Such investigations are important, as induced resistance may be the most important type of resistance that occurs in plants (23). This report apparently is the first one describing induced resistance obtained in plants grown in a gnotobiotic environment. Since the necrotic reaction occurred only in susceptible varieties, it may be possible that a method that utilizes infiltration with bacteria might be devised for screening for resistance to *C. insidiosum*.

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