Differences in Anatomy, Plant-Extracts, and Movement of Bacteria in Plants of Bacterial Wilt Resistant and Susceptible Varieties of Alfalfa

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ABSTRACT

Roots and stems of plants of alfalfa (*Medicago sativa*) varieties resistant to bacterial wilt caused by *Corynebacterium insidiosum*, had fewer vascular bundles, shorter vessel elements, and a thicker cortex than those of plants of susceptible varieties. The number of vessel elements present in vascular bundles, the shape of vessel elements in cross section, and the compactness of xylem cells were not correlated with resistance to wilt. The pathogen grew less profusely in crude aqueous extracts from roots or whole plants, from resistant varieties. Bacteria sprayed onto wounded cotyledons entered and moved more rapidly through plants of susceptible than resistant varieties.

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Bacterial wilt of alfalfa (*Medicago sativa* L.), caused by *Corynebacterium insidiosum* (McCull.) Jens., which has threatened alfalfa production since 1925 (4), is readily controlled by using resistant varieties and the resistance is easily incorporated into new varieties. The resistant varieties apparently remain resistant; the varieties 'Ranger' and 'Vernal', which have been grown extensively over vast areas of the USA and Canada for about 20 years, apparently are as resistant today as when they were released.

Little is known about the nature of resistance to bacterial wilt in alfalfa. Jones (5) and Peltier & Schroeder (8) studied the anatomical characteristics of resistant and susceptible plants; however, they disagreed about the importance of these characteristics. The objective of our investigation was to provide data that might help explain why resistance to bacterial wilt of alfalfa is stable. We compared resistant and susceptible varieties for anatomical differences, determined whether extracts from the two classes of varieties would influence growth of the pathogen, and traced the movement of the pathogen within plants of resistant and susceptible varieties.

MATERIALS AND METHODS.—*Anatomical studies.*—To learn whether modern varieties differ in anatomical characters that might be related to wilt resistance, we studied resistant alfalfa varieties 'Ramsey', 'Ranger', and 'Vernal', and susceptible varieties, 'African', 'Du Puits', Narragansett', and 'Rhizoma' (2). The plants were grown in steamed soil in the greenhouse at 20 C, until they were 7 weeks old, when stems and roots of uniform size were sectioned using a cryostat. Sections taken from the first internode above the cotyledon for stem studies and 1.5 cm below the crown for root studies were mounted in lactophenol on microscope slides.

Thickness of cortex, number of vascular bundles/stem, number of xylem strands/root, number of vessel elements per vascular bundle or strand, shape of vessel elements, and degree of compactness of xylem cells in vascular bundles or strands were observed in each cross section. The length of vessel elements was measured in longitudinal sections; three complete elements were measured in each plant. Fifty plants of each variety were observed for each of those plant characteristics.

Growth of bacteria in plant extracts.—We determined the capacity of *C. insidiosum* to grow in crude extracts prepared from 7-week-old plants of Vernal (resistant), and of the susceptible varieties, African, Du Puits, Narragansett, and Rhizoma. Plants of uniform size, 30 per variety, were washed free from soil and ground in a mortar in 100 ml of sterile distilled water. This crude extract was centrifuged at 7500 rpm in a Servall Angle centrifuge for 10 min to remove debris and then sterilized by filtration through Oxoid membrane filters. The extracts were prepared at 4 C. The sterile extract was placed aseptically in sterile tubes (5 ml/tube) and inoculated with approximately 100,000 bacteria/tube from a pure culture of *C. insidiosum*. Three tubes of extract from each variety were inoculated and three were kept as noninoculated checks. After 72 hr at room temperature (22-24 C), all the tubes of inoculated extracts appeared uniformly turbid. The suspension in the tubes was diluted with sterile distilled water and 1 ml of each of the dilutions, 10^{-4}, 10^{-5}, and 10^{-6}, for each extract was thoroughly mixed into 25 ml of beef lactose agar (at 48-50 C) in petri dishes and kept at room temperature for 96 hr when the colonies were counted. The experiment was repeated twice.

A second experiment was designed to learn the effect of extract from individual plant roots on bacterial growth. The resistant varieties Ramsey, Ranger, and 'Teton' were used, in addition to those listed immediately above. Individual roots of 7-week-old plants (ten/variety) of uniform size, were crushed in a mortar with 7 ml sterile distilled water. Debris was removed and the extract sterilized by filtration through a Millipore filter, pore size

TABLE 1. Range and average number of vascular bundles or strands, length of vessel elements, and thickness of cortex in roots and stems of plants from alfalfa varieties resistant or susceptible to bacterial wilt[a]

Variety	Roots		Stems	
	Range	Avg	Range	Avg
	Number of vascular bundles or strands/plant			
Resistant				
Ramsey	5-8	6.4 a	6-12	8.4 a
Ranger	5-8	6.4 a	6-10	8.2 a
Vernal	5-8	6.9 a	6-11	8.3 a
Susceptible				
African	6-9	7.7 b	6-11	8.8 b
Du Puits	6-10	7.8 b	6-12	9.4 c
Narragansett	6-10	7.2 b	6-12	9.1 bc
Rhizoma	6-10	7.7 b	6-12	9.5 c
	Length (μ) of vessel elements			
Resistant				
Ramsey	104-198	152 a	146-261	189 b
Ranger	94-219	153 a	157-230	183 b
Vernal	104-219	164 b	146-219	175 a
Susceptible				
African	136-251	184 d	157-282	215 d
Du Puits	125-240	175 c	146-261	203 c
Narragansett	125-261	187 d	157-271	214 d
Rhizoma	136-240	186 d	167-271	211 d
	Thickness (μ) of cortex			
Resistant				
Ramsey	136-324	229 a	63-125	99 ab
Ranger	178-324	239 a	63-135	104 a
Vernal	157-313	222 ab	73-135	96 b
Susceptible				
African	94-324	211 bc	63-125	86 c
Du Puits	94-292	200 c	63-94	80 cd
Narragansett	73-230	136 d	53-104	78 d
Rhizoma	115-313	195 c	63-125	86 c

[a] Range and avg values based on 50 plants per variety. Length of vessel elements determined from three elements per plant. Differences in the average values were significant at the 1% level for the number of vascular bundles or strands and the length of vessel elements and at the 5% level for the thickness of the cortex. For each plant characteristic data followed by the same letter are not significantly different according to Duncan's multiple range test.

0.5 - 1.0μ. Two vials, each containing 1 ml of extract from each root, were each inoculated with about 47,000 bacteria and two vials that did not receive bacteria served as noninoculated checks. The vials were kept at room temperature for 96 hr, when the bacteria in one vial were estimated by the dilution-plate method. The experiment was repeated once.

Location of bacteria in plants at time intervals after inoculation.—We employed the cotyledon method of inoculation (7) to investigate the ability of *C. insidiosum* to invade modern varieties resistant or susceptible to the pathogen. The distribution of bacteria from the cotyledons was determined for Du Puits and Narragansett (susceptible varieties) and for Vernal (resistant). Seeds were planted in steamed soil in clay pots in a greenhouse and, when the plants were 8 days old, they were thinned to about 36/pot. A bacterial suspension obtained by soaking 100 g of diseased roots in a liter of tap water for 0.5 hr (6), was sprayed onto the cotyledons until run-off. While the plants were still wet, about one-third of each cotyledon was cut off, and the seedlings were sprayed again with the bacterial suspension and then returned to the greenhouse. The plants in each pot were enclosed in a plastic bag for 24 hr. Five to 15 plants of each variety were taken for sectioning at each of the intervals 0.5, 1, 2, 4, 7, 16, 26, 32, and 60 days after inoculation. Most plants were fixed in FAA (9) and embedded in paraffin prior to sectioning, however, 60-day-old plants were sectioned with a cryostat immediately after collection. Sections 10 to 15-μ thick were made from each plant at 4 to 5-mm intervals beginning at the site of inoculation on the cotyledon and extending to the shoot and root apices. All sections were stained with carbol fuchsin and light green (9) with the exception of the sections made from 60-day-old plants which were stained with gram stain. The location of bacteria in the plant tissues was observed with the microscope at X 1,000.

RESULTS.—*Anatomical studies.*—There were fewer vascular bundles in stems and roots of plants from resistant varieties than of plants from susceptible varieties (Table 1). Average differences between the resistant and susceptible classes of

TABLE 2. Number *Corynebacterium insidiosum* cells in aqueous extracts[a] from alfalfa plants of five varieties after 72 hr at room temperature

Variety	Average no. and range of bacteria ($\times 10^5$/ml) per trial[b]					
	First trial		Second trial		Third trial	
	Avg no.	Range	Avg no.	Range	Avg no.	Range
Resistant						
Vernal	92	3	87	7	72	10
Susceptible						
African	372	20	324	20	369	
Du Puits	330	20	312	20	394	10
Narragansett	283	20	293	20	276	20
Rhizoma	234	7	268	10	253	20

[a] Aqueous extract of 30 plants (roots and stems) of each variety/trial.

[b] Numbers are averages of three plate counts. Original inoculum was about 20,000 bacteria/ml of extract. Differences between Vernal and the other varieties were statistically significant.

varieties were statistically significant but the differences among varieties within each class were not significant.

The vessel elements in roots and stems of plants of resistant varieties were shorter than those of susceptible varieties (Table 1) and the differences between the two classes of varieties were statistically significant. Within each resistance class, there were differences among the varieties. Vessel elements in roots of Vernal averaged 10 μ longer than elements in roots of the other two resistant varieties and vessel elements in Du Puits averaged 10 μ shorter than elements in the other susceptible varieties. The vessel elements in stems of Vernal averaged 8 μ shorter than those in the other two resistant varieties and those of Du Puits averaged about 8 μ shorter than the elements in the other susceptible varieties.

The cortex was thicker in stems and roots of plants from resistant, than from susceptible, varieties (Table 1) and the average differences between the resistant and the susceptible varieties were statistically significant. The cortex of Narragansett was significantly thinner than that of any of the other susceptible varieties.

The number of vessels per vascular bundle was so variable in roots and stems of both resistant and susceptible varieties that the varieties could not be distinguished on this basis. The vessel elements in both roots and stems were round, ellipsoidal, and angular in cross section which precluded distinguishing the varieties in terms of vessel shape. The compactness of xylem cells in vascular bundles appeared to be related to the diameter and number of vessel elements, however, the varieties could not be distinguished by this character.

Growth of bacteria in plant extracts.—Aqueous extracts prepared from a composite of 30 entire plants of each of the susceptible varieties African, Du Puits, Narragansett, and Rhizoma, supported three to four times more bacteria during a 72-hr incubation period than did extracts from a like number of Vernal plants (Table 2).

The numbers of bacteria supported by root extracts of individual plants varied greatly among plants within all varieties (Table 3). A range of about 30×10^5 to more than 300×10^5 cells per ml was observed within all varieties. However, the average numbers of bacterial cells in the extracts from the roots of susceptible varieties ranged from nearly two to more than three times the numbers of cells in the extracts from resistant varieties. The higher numbers of bacteria in extracts from some plants of resistant varieties might be explained by the fact that even resistant varieties may include many susceptible plants. On the other hand it is difficult to explain why so many extracts from plants of susceptible varieties supported low numbers of bacteria, because the susceptible varieties contained fewer than 1% resistant plants. Perhaps we tested too few plants from each variety.

When plant extracts were autoclaved and then inoculated there was no apparent inhibition of bacterial growth in extracts of 10 individual plants from each of the varieties Ramsey, Teton, African, or Rhizoma.

Location of C. insidiosum in plants at time intervals after inoculation.—Twelve hr after the cut surfaces of cotyledons of resistant Vernal and susceptible Du Puits and Narragansett were inoculated, the bacteria had penetrated 1-2 mm into the cotyledon and were associated only with the vascular parenchyma cells (Table 4). In sections from about 100 plants of each variety, in only one instance were bacteria seen in association with mesophyll cells of a cotyledon (Narragansett) soon after inoculation.

One day after inoculation of plants of the susceptible varieties the bacteria were in the vascular parenchyma cells at the base of the cotyledon; i.e., 7 mm from the site of inoculation. Two days were required for the bacteria to appear at a similar location in plants of the resistant variety.

Two days after inoculation the bacteria were seen in vessel elements 10-25 mm below the inoculation site in the hypocotyl of plants from susceptible

TABLE 3. Number of *Corynebacterium insidiosum* cells in aqueous extract from roots of 10 individual plants of eight alfalfa varieties after 96 hr at room temperature

	Avg no. of bacteria ($\times 10^5$/ml) per individual root extract[a]										
Variety	1	2	3	4	5	6	7	8	9	10	Variety avg
Resistant											
Ramsey	62	35	94	397	21	60	338	640	109	31	176.9
Ranger	89	54	469	76	83	50		56	71		118.5
Teton	78	513	97	34	84	93		77	425	355	195.2
Vernal	521	42	671	37	35	25	52		36	22	160.1
Susceptible											
African		303	62	710	88	34	372	510	1,060	19	351.0
Du Puits	592	68		638	93		484	537	693	71	396.3
Narragansett	759	693	68	638	726	85	598	612		683	540.2
Rhizoma	39		496	80	820	49	529	607	730	393	414.8

[a] Numbers are averages of three plate counts. Original inoculum was about 47,000 bacteria/ml of extract. Differences between the resistant and the susceptible varieties were statistically significant at .05. A blank indicates no data obtained owing to contamination.

varieties but they were not seen in a similar location in plants from the resistant variety until 7 days after inoculation and then only in one of 15 plants examined.

At 7 days after inoculation, the bacteria were found in vessel elements and in xylem parenchyma cells at the crown (30-40 mm below the cotyledon) in plants of the susceptible varieties. The bacteria were also seen at this location in two of 15 plants of the resistant variety at that time but usually, they were not seen there until 16 days after inoculation.

Sixteen days after inoculation the bacteria were found in the roots of plants of the susceptible varieties. The bacteria, while in the stems, occurred only in one or two of the vascular bundles. Upon reaching the roots, however, they rapidly spread into all of the vascular bundles. Invasion of the roots of plants of the resistant variety was first noted 26 days after inoculation of the cotyledons and then in only five of 15 plants examined. In the roots of all varieties the bacteria were found only in vessel elements and vascular parenchyma cells.

Bacteria were not seen in tissues above the inoculated cotyledons of plants from susceptible varieties until 16 days after inoculation, and after the bacteria had become established in the root system. The bacteria were found in the first, second, and fourth internode above the cotyledon 16, 26, and 32 days, respectively, after inoculation. About the 60th day after inoculation and thereafter, the bacteria were found in the vessel elements throughout the susceptible plant, except in newly formed shoots.

TABLE 4. Location *Corynebacterium insidiosum* in organs and tissues of plants of resistant 'Vernal' and susceptible 'Du Puits' and 'Narragansett' alfalfa varieties at different times after inoculation of cotyledons of 8-day-old plants

Days after inoculation	Bacteria observed in:[a] Organs	Tissues & cells	Distance (mm) from inoculation site
		Resistant variety	
0.5	Cotyledon	Xylem parenchyma	1-2
1	Cotyledon	Xylem parenchyma	2-3
2	Cotyledon	Xylem parenchyma	5-7
4	Cotyledon	Xylem parenchyma	7-10
7	Hypocotyl	Xylem parenchyma + a few vessels	15-25
16	Crown	Xylem parenchyma + a few vessels	30-40
26	Root	Parenchyma and vessels	50
32	Root	Parenchyma and vessels	50
60	Root	Parenchyma and vessels	50
		Susceptible varieties	
0.5	Cotyledon	Xylem parenchyma	1-2
1	Cotyledon	Xylem parenchyma	7
2	Hypocotyl	Xylem parenchyma + a few vessels	15-25
4	Hypocotyl	Parenchyma + most vessels	25-30
7	Crown	Parenchyma and vessels	30-40
16	Root and stem	Parenchyma and vessels	50 and 1st stem internode
26	Root and stem	Parenchyma and vessels	2nd internode
32	Root and stem	Parenchyma and vessels	4th internode
60	Root and stem	Parenchyma and vessels	All over

[a] Observations based on 5-15 plants per variety.

Bacteria were seen in stems above inoculated cotyledons of plants from the resistant variety in only four of 48 plants examined.

A dark, gumlike substance formed in the vessel elements in roots and stems of most plants of the susceptible varieties after the cells were populated with bacteria, about 60 days after inoculation. Gum was observed in roots, but not stems, of plants of the resistant variety but only for those few plants in which bacteria were found.

DISCUSSION.—The control of bacterial wilt of alfalfa is easily accomplished by means of resistant varieties and provides a classic example of disease control by that method. It is possible that resistance to bacterial wilt in alfalfa is an excellent example of horizontal resistance as discussed by Van der Plank (10). The resistant varieties Ranger and Vernal have retained their resistance for many years even though they have been grown in many different environments and have been exposed to many different populations of the *C. insidiosum*. Our data indicate that, in resistant varieties, both morphological and physiochemical barriers retard and prevent wilt development. This suggests that resistance may be controlled by several genetic factors.

Varieties homozygous for wilt resistance are not available. All alfalfa varieties are extremely heterogeneous; some plants of a variety are wilt-resistant, whereas others are susceptible. None of the varieties that we studied were comprised only of resistant plants. Plants of alfalfa varieties are probably just as variable for other characters as for wilt resistance. To permit valid conclusions, we studied as many plants of a variety as was feasible. However, the number used was necessarily small. A more reliable sample for each treatment would have entailed examinations of about 100 plants per variety.

Not much is known about the nature of wilt resistance in alfalfa nor has it been studied much, probably because most of the effort has been made in developing resistance. Peltier & Schroeder (8) thought resistance was due to anatomical differences between roots of resistant and susceptible varieties. They reported that vessel elements were shorter, smaller in diameter, and had thicker walls, and that the vascular strands and vessel elements were more widely separated from each other by masses of parenchyma cells in resistant varieties than in susceptible varieties. They also noted that the bacteria were restricted largely to vascular tissue in resistant varieties but not in susceptible varieties. They concluded that the morphological features of the resistant root inhibited rapid development of the bacteria and their invasion of the vital tissues. Jones (5) did not find structural differences between resistant and susceptible varieties. He did find, however, that bacteria in resistant varieties were largely restricted to the vascular parenchyma and he believed that the factor that controlled wilt resistance was associated with that tissue.

We found both morphological and physiochemical barriers that might retard and prevent wilt development. There were fewer vascular bundles, shorter vessel elements in vascular bundles, and a thicker cortex in plants of resistant varieties than in susceptible varieties and the cell sap extract from more plants of resistant varieties than of susceptible varieties inhibited the growth of the pathogen in vitro. Because *C. insidiosum* is a vascular pathogen, each of these plant characteristics might make it more difficult for infection to occur and would tend to slow the multiplication and movement of the bacteria once they had gained entry. Thus, small numbers of vascular bundles more deeply embedded in cortical tissue should be less likely to become infected than numerous vascular bundles covered with a thin cortex. Furthermore, the pathogen should have more difficulty moving through short vessel elements than through long ones because of the presence of many more obstructions at the point of union of the several elements. In addition, if the cell sap of plants from resistant varieties has the same inhibitory properties in vivo as were indicated in vitro, the pathogen probably finds itself in an environment unfavorable for growth as it passes through the cortex and vascular tissue of plants of resistant varieties. All of our work suggests that more inoculum and a longer time for disease to develop are required for wilt development in resistant varieties than in susceptible varieties.

Hawn & LeBeau (3) and Cormack et al. (1) found that alfalfa tissue steeped in water released an antibiotic substance that inhibited the growth of *C. insidiosum* in vitro. Apparently an antibiotic was produced by microorganisms associated with the alfalfa tissue. They thought the antibiotic might be involved in the resistance of alfalfa to wilt because more inhibition of *C. insidiosum* was obtained with preparations from resistant varieties than from susceptible varieties. It is possible that the substance that inhibited growth of the bacteria in the extracts we obtained from alfalfa was the same as that obtained by Hawn & LeBeau or Cormack et al. However, we think that another factor may be involved because we obtained our extracts at temperatures at which there should have been little microbiological activity and therefore, less opportunity for an antibiotic to be produced while the extracts were being prepared. We believe that resistant plants may contain a heat labile antibacterial substance that reduces the growth of the bacteria and thereby is involved in resistance of alfalfa to wilt. Our work merely suggests the occurrence of such a substance and more detailed experimentation should be done.

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