Influence of *Corynebacterium insidiosum* on Water Relations of Alfalfa

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ABSTRACT

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The effect of *Corynebacterium insidiosum* on the water relations of alfalfa was studied in a 6-yr-old stand of Ranger alfalfa. Stomatal conductance, xylem pressure potential, and root, stem, and leaf liquid flow conductance were measured periodically. At all times, the xylem pressure potential was more negative in diseased than in healthy plants. Stomatal function was not impaired in either diseased or healthy plants. A 60-fold decrease in liquid flow conductance through petioles and leaflet veins of diseased plants probably was the cause of water stress. Flow of water through other parts of the vascular system of diseased plants was only slightly impaired. No evidence of cellular membrane damage was found in water regain or electrolyte leakage studies. The location of impaired water movement was not correlated with the location of the largest numbers of bacteria.

Bacterial wilt of alfalfa (*Medicago sativa* L.) induced by *Corynebacterium insidiosum* (McCull) H. L. Jensen is characterized by stunting, reduced vigor, yellowing, wilting, and eventually death of infected plants. The manner in which bacteria affect the plant's water relations to induce such changes is not known. Generally, plant pathogens cause water imbalance by interfering with water movement in the plant (10,11) or with stomatal regulation or by affecting the water-holding ability of cellular membranes (23).

In bacterial wilt of alfalfa, the large numbers of bacteria found in the crown region generally have been thought to be responsible for interference with water movement in the plant (18). Ries and Strobel (19,20), however, reported that a glycopeptide produced by the bacteria in diseased plants acts as a phytotoxin. Van Alfen and Turner (25) reported that this glycopeptide interferes with water movement through the plant's vascular system, and quantities required to wilt alfalfa shoots were found within diseased plants.

There has been much speculation concerning the mechanisms by which vascular pathogens are able to induce water stress in their hosts (2,4,9,15,21,22). Few studies have been done, however, to definitely determine the role of the pathogen (7,10,11,13,22). Our study on bacterial wilt of alfalfa was initiated to better understand how bacteria affect the host plant's water balance. We were specifically interested in knowing whether water stress resulted from excess loss of water or from interference with water movement within the plant and whether impaired movement could be attributed to bacteria.

MATERIALS AND METHODS

Two diagonal transects 224 m long were made across a 6-yr-old stand of Ranger alfalfa in Smithfield, UT, and 25 locations along the transects were chosen randomly. Near each location, a plant with symptoms of bacterial wilt of alfalfa and a healthy control plant were selected. The plants were marked for easy identification during subsequent measurements.

Soil moisture measurements were taken five times during a 2-wk period by the soil auger method. Since alfalfa is a deep-rooted plant, soil moisture was measured approximately every 15 cm, down to 150 cm. For each group of samples collected at a given time, five replications were taken from five well-scattered points along the transects. Soil water contents were determined gravimetrically. During the period of study, the field was not irrigated and no rainfall was recorded. The temperature varied within a range of 21–33 C and the relative humidity values were generally 32–54%.

During the 2 wk, the 25 pairs of diseased and healthy plants were measured periodically to determine stomatal conductance, xylem pressure potential, and liquid flow conductance. Stomatal conductance was determined with a Delta T automatic porometer (Delta T Devices, Burwell, Cambridge, England). The original area of the Delta T sensor slit was decreased from 0.56 to 0.14 cm^2 to fit the small alfalfa leaves. Leaves were picked from approximately the same position in the plants, since stomatal resistance is markedly affected by irradiance. The porometer was calibrated at 22, 27, and 31 C, temperatures representative of those in the field. Porometer readings were taken in a relative humidity range of 32–52%, as determined by a sling psychrometer.

Xylem pressure potential (Ψ) was measured by the pressure chamber method (1,11) (PMS Instrument Company, Corvallis, OR). Pressure chamber measurements were not calibrated against a thermocouple psychrometer but were assumed to approximate leaf water potential (3,12).

To determine the water potential at which plants wilt visibly, xylem pressure potentials were measured in eight pairs of healthy and diseased plants. The plants were then uprooted and progressively dried. Measurements were taken when 50, 75, and 100% of the leaves wilted. The diseased plants were about 25% wilted initially.

Conductance of the plant's vascular system to flow of water was determined with a pressure chamber (24,25). The stem was cut approximately 10 cm from the top of the plant. The stem below this cut was divided into two parts, the top 15 cm and the lower 15 cm (Fig. 1), and 10-cm segments were cleanly cut with a sharp razor blade from both parts (0.2–0.3 cm diam). The segments then were sealed into the pressure chamber, with the end inside the chamber dipping into a 5-ml test tube containing 3 ml of filtered, degassed, sterilized distilled water. Any leaves were severed flush to the stem, and the stem was placed in the pressure chamber so that the wounds were submerged in water. A short, small-diameter tube filled with sterile water and attached to a horizontal 0.1-ml pipet was connected to the other end of the stem, outside the chamber. The time for each 0.01 ml of water to flow from the stem under a constant pressure of 2 bars was recorded until 0.08 ml had been collected. The vascular fluid thus collected was plated on yeast extract-dextrose-calcium carbonate (YDC) agar (19).

The same procedure was used for the crown region and the uppermost part of the taproot, except a 1-ml pipet was used and the flow rate of each 0.1 ml was determined. Crown regions were 10 cm long and 0.5–0.7 cm in diameter at the top. Taproots 10 cm long were taken 15–17 cm below the crown region. A constant pressure

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of 1 bar was applied.

Conductance was determined on petioles 0.1 ± 0.4 cm in diameter and 1.7 ± 0.1 cm long, as described for stem conductance.

Shoots 10 cm long and about 0.2 cm in diameter were used to determine liquid flow conductance through leaf veins. One quarter of the tip was cut off the terminal leaflet of the lowermost leaf. A constant pressure of 2 bars was applied, and the liquid exuding through the cut leaf veins was collected on a previously weighed filter paper and quickly sealed in a small vial. The volume of water exuded was determined by weight.

The number of *C. insidiosum* within diseased and healthy plants was determined in 10 pairs of healthy and diseased plants. Three surface-sterilized samples each from the crown region, petioles, and terminal leaflets were ground in a sterile TenBroek homogenizer (Kontes, Vineland, NJ). The resultant plant tissue suspensions were diluted and plated on YDC agar (19) and Difco beef-lactose agar (BLA). Colonies were counted after 4–6 days of incubation at 20 C.

Membrane integrity of healthy and diseased plants was assessed by a water regain method (14). Six pairs of diseased and healthy plants were dug up after their Ψ and leaf conductance were determined in the field. The plants were kept with the roots in water and taken to the laboratory. To reduce turgidity, stems were cut and the plants were allowed to dry slightly. Three leaf discs 0.4 cm in diameter were then cut from each plant. The discs were floated on water in a closed petri dish at 25 C, taken out immediately, blotted dry, and weighed. The discs then were refloated and periodically weighed. Control leaf discs were taken from healthy plants, frozen for 4 hr, thawed, then floated and treated as described. The discs were dried at 80 C and weighed.

Membrane integrity also was tested by following changes in electrolyte leakage of 0.4-cm leaf discs floated on distilled water (8). Electrolyte leakage was estimated with a Markson Electro Mark (Del Mar, CA) conductivity meter.

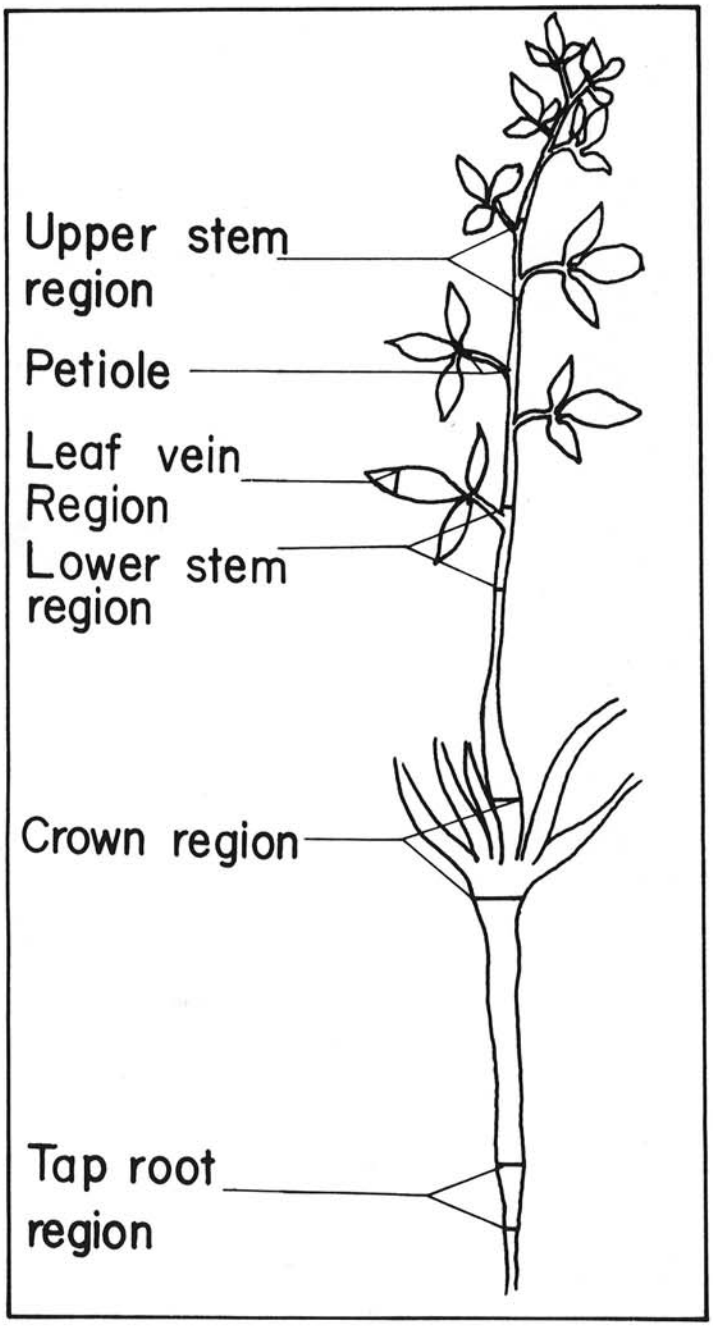

Fig. 1. Locations from which samples were taken for studies of water relations in alfalfa plants.

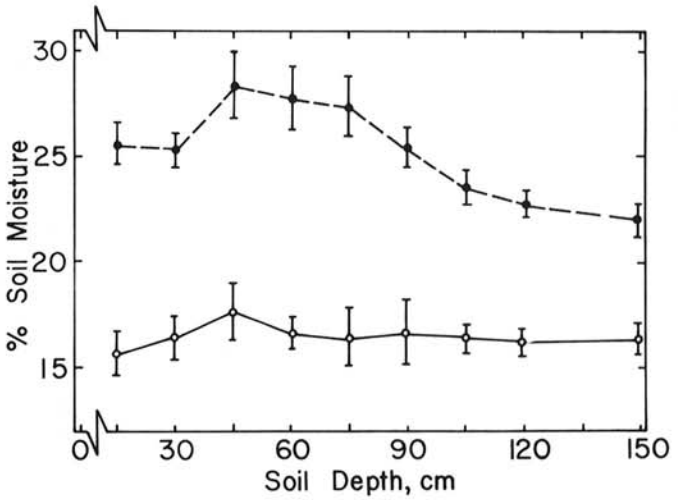

Fig. 2. Initial (●) and final (○) percentages of soil moisture at soil sample depths. Each point represents the mean of five soil moisture measurements well scattered in the field. Vertical lines represent ± standard error.

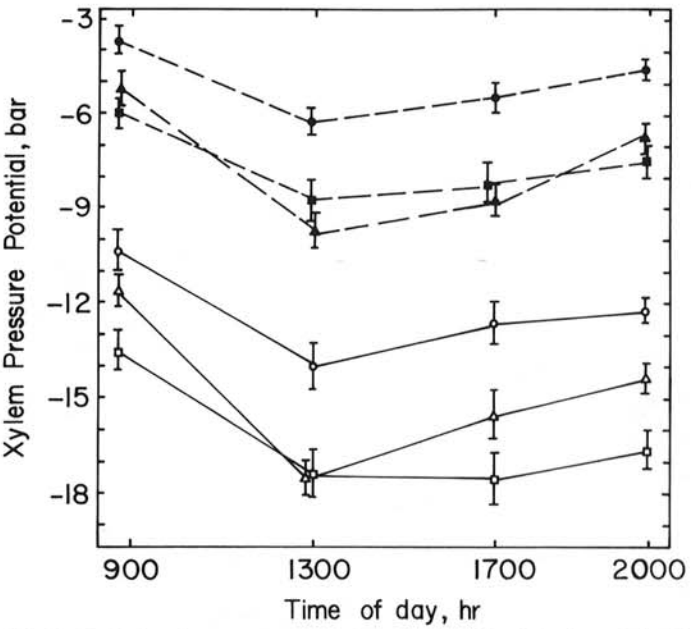

Fig. 3. Periodic xylem pressure potentials of pairs of healthy plants (closed symbols) and plants infected with *Corynebacterium insidiosum* (open symbols) taken throughout day 1 (○), day 7 (△), and day 15 (□). Each point represents the mean of five plants well scattered in the field. Vertical lines represent ± standard error.

RESULTS

The soil in the alfalfa field varied from a mildly to strongly alkaline silt loam and loam in the upper surface layers to a very fine sandy loam below a depth of about 150 cm. Initially, the soil water content was 30–35%, w/w. By the end of 2 wk, all areas showed an appreciable decrease in soil moisture (Fig. 2). The soil finally reached a range of 14–20% water content, which is close to the wilting point for plants.

Xylem pressure potentials of diseased plants were significantly less than those of healthy plants. As soil moisture decreased, the Ψ of healthy plants dropped but that of diseased plants dropped even more (Fig. 3). Predawn leaf water potentials of healthy and diseased plants were considerably different. Even though Ψ decreased to -11 bars at midday in healthy plants, it recovered to essentially zero during the night. In diseased plants, however, there was very little recovery at night and predawn Ψ averaged -17.08 ± 0.33 bars even though relative humidity was 90–93%.

The fact that diseased plants wilted at a lower Ψ than healthy plants (Fig. 4) is evidence that they made some adjustment to a continued state of water stress. At 100% wilt, healthy plants had a Ψ of -16.5 ± 0.2 bars and diseased plants a Ψ of -19.0 ± 0.3 bars.

Stomatal conductance was always less in diseased plants than in healthy plants (Fig. 5). As the soil dried and the Ψ became more negative, stomatal conductance decreased much more in diseased plants than in healthy ones, probably because of water stress.

Stomatal conductances measured 4 hr after sunset were 0.02 and 0.01 cm sec^{-1} for diseased and healthy plants, respectively. In healthy plants, stomatal conductance increased from 0.01 cm sec^{-1} at night to 0.15–0.28 cm sec^{-1} during the day, then decreased again to near zero at night. In diseased plants, stomatal conductance was 0.02 cm sec^{-1} at night and 0.11–0.17 cm sec^{-1} during the day.

In all cases, liquid flow conductances for the vascular system of the lower and upper stems, crown region, taproot, petioles, and leaflet veins were higher in healthy plants than in diseased ones (Table 1). For both healthy and diseased plants, conductance was highest in the taproot region and lowest in leaflet veins. Conductance through the stem-petiole-leaflet veins was almost 60 times less in diseased plants than in healthy ones (Table 1). Because the decreases in relative conductance of petioles and stems of diseased plants were small when conductance through these parts was measured separately, the large increase in resistance through the entire system must have been located either in the node at the petiole-stem junction or in the leaflet veins.

The bacteria in the vascular fluid were gram-positive, nonmotile, and rod-shaped. Colonies were reddish pink on tetrazolium chloride agar (6) and blue-black on BLA (5), characteristic of *C. insidiosum*. Pathogenicity tests (5) confirmed the presence of *C. insidiosum*. On a fresh weight basis, there were 1.1×10^5 bacteria per gram in leaflets, 3.4×10^5 bacteria per gram in petioles, and 2.2×10^9 bacteria per gram in the crown regions of diseased plants plated on YDC agar (Table 2). All healthy plants were free of bacteria.

TABLE 1. Comparative liquid flow conductance values from different portions of healthy alfalfa plants and plants infected with *Corynebacterium insidiosum*

Sample area	Conductance ($\times 10^{-4}$ $cm^3 bar^{-1} sec^{-1}$)[a]		Healthy: diseased ratio
	Healthy	Diseased	
Taproot	80.8 ± 2.4	57.8 ± 1.6	1.40
Crown	66.2 ± 1.8	46.8 ± 0.8	1.43
Lower stem	2.43 ± 0.05	1.83 ± 0.04	1.37
Upper stem	2.27 ± 0.04	1.73 ± 0.02	1.31
Petiole	1.46 ± 0.02	0.62 ± 0.01	2.34
Stem-petiole-leaflet	0.14 ± 0.01	0.0024 ± 0.0014	60.0

[a]Means ± standard error of conductance values from 10 plants.

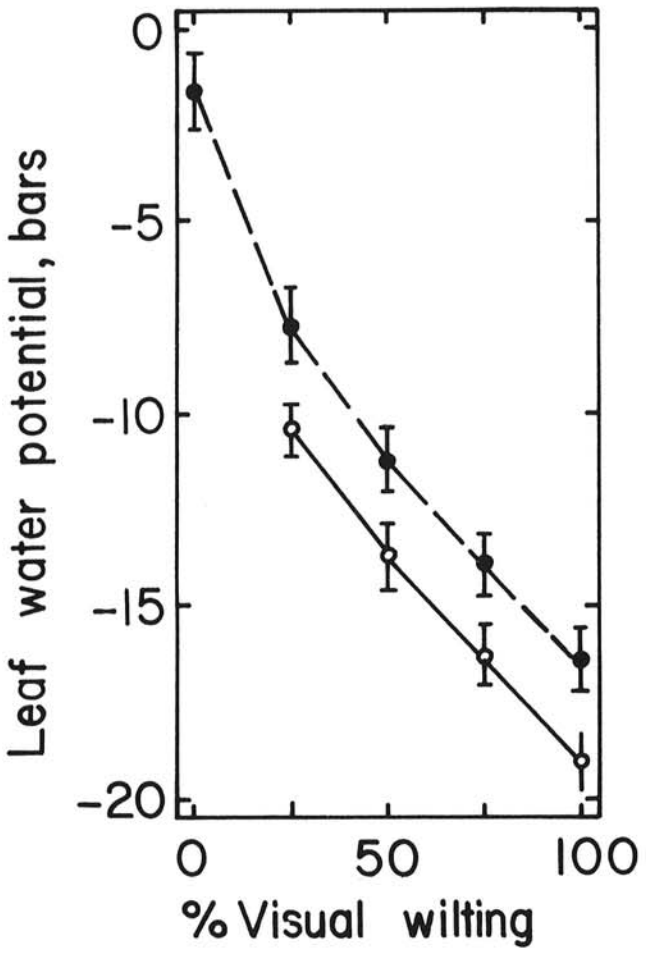

Fig. 4. Xylem pressure potential at which healthy plants (●) and plants infected with *Corynebacterium insidiosum* (○) were visually rated at 0, 25, 50, and 100% wilted. Each point represents the mean of eight plants. Vertical lines represent ± standard error.

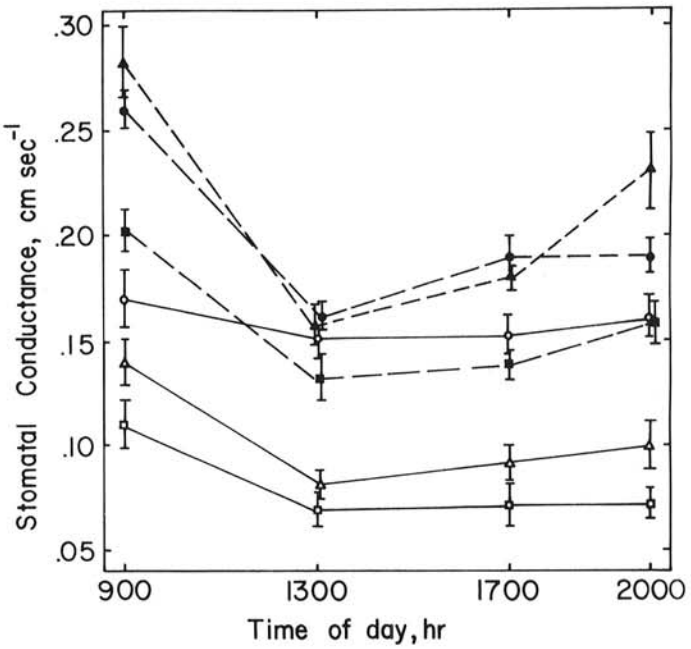

Fig. 5. Periodic stomatal conductance of pairs of healthy plants (closed symbols) and plants infected with *Corynebacterium insidiosum* (open symbols) taken throughout day 1 (○), day 7 (△), and day 15 (□). Each point represents the mean of five plants well scattered in the field. Vertical lines represent ± standard error.

TABLE 2. Populations of *Corynebacterium insidiosum* in different parts of diseased alfalfa plants

Plant part	Medium[a]		
	YDC	BLA	TTC
Leaflet veins	$1.1 \pm 1.37 \times 10^5$[b]	$0.77 \pm 0.78 \times 10^5$	$0.3 \pm 0.48 \times 10^5$
Petioles	$3.4 \pm 1.25 \times 10^5$	$3.1 \pm 1.65 \times 10^5$	$2.6 \pm 1.8 \times 10^5$
Crown region	$2.24 \pm 2.32 \times 10^9$	$2.1 \pm 3.5 \times 10^9$	$1.8 \pm 1.8 \times 10^9$

[a]YDC = yeast extract-dextrose-calcium carbonate agar; BLA = Difco beef-lactose agar; TTC = tetrazolium chloride agar.
[b]Data represent mean ± standard error of 30 samples.

TABLE 3. Water uptake of leaf discs from healthy alfalfa plants and plants infected with *Corynebacterium insidiosum*

Sample	Increase in fresh weight[a] (mg)			Oven dry weight (mg)
	0 hr	24 hr	32 hr	
Healthy	2.7 ± 0.1[b]	3.8 ± 0.2	3.9 ± 0.1	1.3 ± 0.1
Diseased	2.1 ± 0.1	3.2 ± 0.1	3.02 ± 0.2	0.75 ± 0.2
Frozen and thawed[c]	1.3 ± 0.1	1.5 ± 0.04	1.63 ± 0.08	0.48 ± 0.2

[a]Alfalfa stems with attached leaves were allowed to dry to reduce turgor, then 0.4-cm diameter discs cut from the leaves were floated on water in a closed petri dish at 25 C.
[b]Each value is the mean ± standard error of 18 discs.
[c]To disrupt membranes as a control, discs from healthy plants were frozen, then thawed before being floated on water.

Although the equilibrium weights of leaf discs differed in healthy and diseased plants (Table 3), discs from diseased plants showed substantial water regain after drying. The difference in equilibrium weights may have been due to a lower capacity of diseased plants to regain water as reflected in the dry weight differences of the two sources of discs. Frozen and thawed discs showed little water regain and had the lowest dry weight. Since these discs were taken from healthy plants, the difference in dry weight between the two samples was probably due to loss of cellular contents when the discs were floating in water. Electrolyte studies confirmed that losses from discs of healthy and diseased leaves were slight in all cases, suggesting that cellular membrane damage is not a factor in water stress of diseased plants.

DISCUSSION

In this study, the xylem pressure potential was much more negative in diseased alfalfa plants than in healthy ones. Diseased plants are under continual water stress, even when watered. For example, the predawn water potentials showed that although healthy plants make an almost complete recovery from stress during the night, diseased plants do not. The leaves of diseased plants were stunted and yellow, but not much wilting was visible. Thus, the stunted appearance of diseased plants probably resulted from low leaf water potential, even though the potential was not low enough to induce continuous wilt.

Due to constant water shortage, a drought hardening effect in the plants was indicated. This is similar to the findings of Dimond and Waggoner (10) on nonsymptomatic leaves of *Fusarium*-infected tomato plants. They found that the stomata of diseased leaves remained closed most of the time. This agrees with our findings that stomatal conductance of leaves to water vapor was higher in healthy plants than in diseased ones during the day. We also found that stomatal closure in diseased plants was not measurably impaired, since stomata responded to low leaf water potentials and closed at night.

The highly negative Ψ in diseased plants could result from mechanical plugging of the vascular system of the root and crown region where the largest numbers of bacteria are. Conductance was decreased by only 1.4-fold in this region in diseased plants, however, and this is not sufficient to cause the observed water stress. The diseased leaflet veins probably are the sites at which resistance to water movement has an impact. Part or all of the resistance, however, may have occurred at the node between the stem and petiole. Van Alfen and Allard-Turner (26) have shown that the vascular systems of the leaflet veins and petiole junctions are the most susceptible to mechanical plugging. The 60-fold reduction of liquid flow of diseased plants over that of healthy plants in this region could have resulted in the low water potentials recorded for diseased plants. Such interference with water movement could keep the leaves continually stressed for water. Other reports indicate that as much as a 200-fold decrease in water movement is observed in some wilt diseases (16,22). Liquid conductance through the crown region was only slightly reduced in diseased plants, in spite of large concentrations of bacteria. This finding is in contrast to some other bacterial wilt diseases in which water stress results from bacterial plugs (17).

No evidence was found that cellular membrane damage is a factor in water stress of diseased plants. The stomatal behavior and adjustment to water stress of diseased plants would require intact membranes. Water stress in alfalfa plants infected by *C. insidiosum* is thus evidently induced by disruption of water movement through the plant's petiole junction or leaflet veins. How bacteria cause this reduction in water movement in this region, the site of lowest concentration of bacteria, is not clear. Possibly, factors other than the physical presence of bacteria in the vessels of these regions are responsible for the interference with water movement. Plants are very susceptible to plugging of their vascular system by macromolecules (26), which can be of host or pathogen origin.

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