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Role of Pit Membranes in Macromolecule-Induced Wilt of Plants[1]

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ABSTRACT

Macromolecules present in low concentrations in xylem fluid of *Medicago sativa* L. var DuPuits will increase the resistance to xylem liquid flow. This increase in resistance was found to be reversible by back-flushing the xylem. Autoradiography showed that very large molecules do not pass through pit membrane pores. A comparison of pit membrane pore sizes to molecule sizes suggests that increased resistance to xylem flow is a result of plugging pit membrane pores. It was also found that pit membranes located in two parts of the plant differ in the apparent diameter of their pores and, thus, in their susceptibility to plugging by macromolecules. Macromolecules in xylem fluid may result from host-parasite interactions and may play a significant role in the outcome of the interaction.

Macromolecules introduced into plant xylem cause an immediate increase in resistance to water flow. This resistance depends on both the size of the introduced macromolecule and the part of the plant through which flow occurs. The cause of this increased resistance is not known, but we can postulate that it is due either to a reactive biochemical mechanism or a physical plugging of pit membranes (3).

Known reactive mechanisms that increase xylem resistance are tylose formation (5) and gelation (15). Both mechanisms are thought to be involved in the resistance of plants to vessel-invading pathogens (5, 15) and to be factors in the dysfunction of vessels with age (9). Neither of these plant responses can explain the instantaneous increase in resistance observed when macromolecules are introduced into plant xylem. Both gelation and tylose formation occur over periods of hours or days (5, 15) rather than the seconds observed with macromolecule-induced xylem flow resistance (13).

Pit membranes, through which water in the transpiration stream must pass, function as microfilters within xylem. These microfilters, while not interfering with the free flow of water, prevent air embolisms and pathogens from passing between vessels (16). Within alfalfa stems, we have found that the average effective vessel length is 30 mm (R. Greer, H. H. Wiebe, N. K. Van Alfen, unpublished). Thus, approximately every 30 mm in the pathway of water movement in stems there are pit membranes that must be traversed by the transpiration stream. If the pores within these membranes are small enough, they could become plugged by macromolecules dissolved in the transpiration fluid, preventing or reducing the passage of water. Data presented here support the hypothesis that macromolecules plug the microfilter-like pores in pit membranes and thus increase xylem resistance. We also show that the apparent diameters of pit membrane pores vary with location within the plant.

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MATERIALS AND METHODS

Xylem Resistance Measurements. Greenhouse grown alfalfa (*Medicago sativa* L. var DuPuits) was used in all studies. The uppermost five or six nodes of alfalfa stems were used before flowering. Below these nodes secondary cambium and hollow pith develops. Xylem conductance measurements were made using a pressure chamber (Soil-Moisture Equipment Corp.), as previously described (4).

The basal ends of stems were placed in solutions of dextran (0.8 g/l) (Pharmacia Fine Chemcials) within the pressure chamber. The dextran solutions were forced through the xylem under 2 bars pressure and the effluent was collected at the distal end of the stems. To collect the effluent from leaf traces, the leaf was removed leaving a small stub of the petiole. A short piece of tygon tubing of known volume connected the petiole stub to a pipet. The connecting tubing contained distilled H_2O so that flow measurements could be taken without delay. Dilution of the effluent with the water was considered in calculations of dextran concentrations. Dextran concentrations in the effluent were determined using the anthrone method (10).

Dextrans through Filters. The ability of dextran solutions to pass through pores of known diameter was determined by forcing dextran solutions (0.5 g/l) under 0.7 bars pressure through membrane screen filters. Both nitrocellulose (Millipore) filters and polycarbonate (Nucleopore) filters of various pore sizes were used. The membranes were held in a Millipore stirred cell holder. Both the flow rate and amount of dextran passing through the filters was determined. Dextran concentrations were determined by the anthrone method (10).

Back-Flushing Stems. To determine whether back-flushing a stem would decrease macromolecule-induced xylem resistance, we introduced 2×10^6 mol wt dextran into 5-cm-long stems of known xylem conductivity until maximum resistance to flow occurred. Half of the stem segments were then back-flushed with water by reversing the direction of xylem flow. The other half were flushed with water in the original, normal orientation. A set of stems was also flushed in both directions with water as a control.

Autoradiography. [^{3}H]dextran was produced as the enzymic product of [^{3}H]sucrose, with the culture filtrate of *Leuconostoc mesenteroides* (ATCC 10830a) being the source of enzyme (7). For autoradiography experiments, alfalfa stems were cut with a razor and quickly recut under filtered, degassed water. Alfalfa shoots were placed in 0.5 g/l solutions of the [^{3}H]dextran and

placed in a growth chamber until the shoots wilted. Wilting occurred within 1 h. Shoot pieces were fixed in gluteraldehyde (2%)-acrolein (3%) dissolved in 0.2 M cacodylate buffer (pH 7.2). The tissue was then washed with this buffer and placed in 1% osmium tetroxide for 2 h. After washing again with buffer, the tissue was dehydrated in a graded series of ethanol followed by acetone. The tissue was embedded in Spurr's resin and then sectioned. Kodak A-10 stripping film was used for light microscope level autoradiography.

Scanning Electron Microscopy. Samples for SEM[2] were cut, fixed in 3% gluteraldehyde, dehydrated in a graded series of ethanol followed by acetone and then critical point dried. The specimens were mounted on stubs, coated with a layer of gold, and viewed with SEM.

RESULTS AND DISCUSSION

Back-Flushing Stems To Decrease Macromolecule-Caused Resistance. If pit membranes become plugged by macromolecules, resulting in increased resistance to xylem conductance, then back-flushing the stems should wash some of the macromolecules out of the pit membrane pores, resulting in a decrease in xylem resistance to water flow. Back-flushing with water increased the conductivity, but the conductivity of control stems remained essentially unchanged (Table I). This back-flushing of stems to increase flow rate is similar to the increase in flow rate that occurs when macromolecules are back-flushed from microfilters.

Measurement of Pit Membrane Pore Sizes. Effective pore diameters of pit membranes, determined by physical means in a number of woody plants, are species specific and vary from 0.056 to 0.84 μm (12). The best methods for measuring pore diameters in plants require application of pressure gradients too steep for use with herbaceous tissues. However, we were able to measure effective pore diameters within alfalfa stems and leaf traces using the ultra-filtration method (11), which is based upon determining pore sizes by the size of particles/molecules that can pass through the pores. Dextrans were used in measuring pore sizes within alfalfa pit membranes. To study the behavior of the dextrans as they pass through pores, we used membrane filters with known pore diameters. The behavior of two dextrans as they pass through the filters is shown in Figure 1. The larger dextran (2×10^6 mol wt) greatly reduced the flow rate (Fig. 1) through all the filters except those with the largest and smallest diameters tested, 1.0 and 0.05 μm, respectively. The macromolecule passes freely through 1.0-μm pores but not through the 0.05-μm pores (Fig. 1). Flow rate through the smaller pore size is not affected presumedly because the dextran is too large to enter and plug the pores. Flow through the largest pore size is not affected because the pore is large enough for the molecule to pass freely. In all of the other pore sizes tested, the 2×10^6 dalton dextran affected flow rates even when most of the dextran molecules were able to pass through. Likewise, the 5×10^5 mol wt dextran reduces flow rate through 0.05 and 0.1 μm pores (Fig. 1), yet about 80% of it is still able to pass through (Fig. 1). This dextran passes freely through pore sizes above 0.1 μm and does not significantly reduce flow rates.

The RMS radii of these two dextrans are larger than would be predicted from their ability to pass through the filters (8). A large proportion of the 2×10^6 mol wt dextran in solution was able to pass through a 0.22-μm filter, yet its RMS radius is 0.34 μm. Dextrans are not rigid, spherical molecules, but behave in solution as branched linear molecules (8). Experimentally, even though they eventually passed through pores smaller than predicted by their RMS radii, the dextrans decreased rate of flow. The only pores through which flow was essentially unaffected (Fig. 1) were those larger than the diameter of the molecule, *e.g.* 1.0 μm pore for the 2×10^6 mol wt dextran, and one so small (0.05 μm) that none of the larger dextran passed through.

Knowing the behavior of dextrans as they pass through pores of known diameter, we can then repeat the flow reduction and molecule retention experiments with alfalfa shoots, instead of membrane filters. The values obtained from introducing both dextrans into stems and leaf traces are shown as points on the curves for the behavior of dextrans through the filters (Fig. 1). Most of both dextrans in solution passed through the alfalfa stems. The 2×10^6 mol wt dextran caused a reduction in flow rates through stems, although 88% of it passed through. Very little of the 2×10^6 mol wt dextran passed through the leaf trace, and it completely stopped flow through this part of the plant's vascular system. About 85% of the 5×10^5 mol wt dextran passed through the leaf trace, yet the flow rate was significantly reduced. These values for dextran behavior in stems and petiole junctions can be used to estimate effective pore sizes by plotting them on the respective lines representing the behavior of the dextrans on membrane filters. When plotted, as shown in Figure 1, the effective pore sizes of pit membranes are about 0.2 to 0.8 μm in stems and about 0.1 μm in petiole junctions. The differences between the estimated pore sizes in leaf traces and stems (0.1 and 0.2 μm, respectively) appear to be significant. The flow of the dextrans through filters are each affected very differently by these two pore sizes as shown by the curves in Figure 1. The only curve that can be used to accurately estimate pore sizes between 0.2 and 1.0 μm diameters is the one representing 2×10^6 D dextran effects on flow rates through filters. Using this curve, the estimate for pore sizes in stems is about 0.5 μm. One of the limitations of this method is that it tends to overestimate pore sizes (11), so these should be considered maximum values only.

Within alfalfa shoots, we have observed two different types of vessesl, one having pitted secondary wall thickenings with round to oval pit membranes. The pit membranes of these vessels have visible holes that vary in diameter (Fig. 2). The average diameter of these holes, measured perpendicular to the long axis of the

[2] Abbreviations: SEM, scanning electron microscopy; RMS, root-mean square.

Table I. *Back-flushing of Alfalfa Stems To Decrease Macromolecule-Caused Resistance*

	Step 1	Step 2	Step 3
Treatment 1:	Water (100%)[a] → Water (103 ± 5[b]%)	→ Direction of flow reversed	→ Water (111 ± 8%)
Treatment 2:	Water (100%) → Dextran[c] (59 ± 4%)	→ Direction of flow reversed	→ Water (82 ± 4%)
Treatment 3:	Water (100%) → Dextran (65 ± 5%)	→ Direction of flow normal	→ Water (67 ± 6%)

[a] Water or dextran (800 μg/ml) was forced (2 bars) through stems (5 cm long). All conductance values were normalized to the initial flow rate of water, expressed as 100%.

[b] Data expressed as mean ± SE.

[c] Dextran was 2×10^6 mol wt.

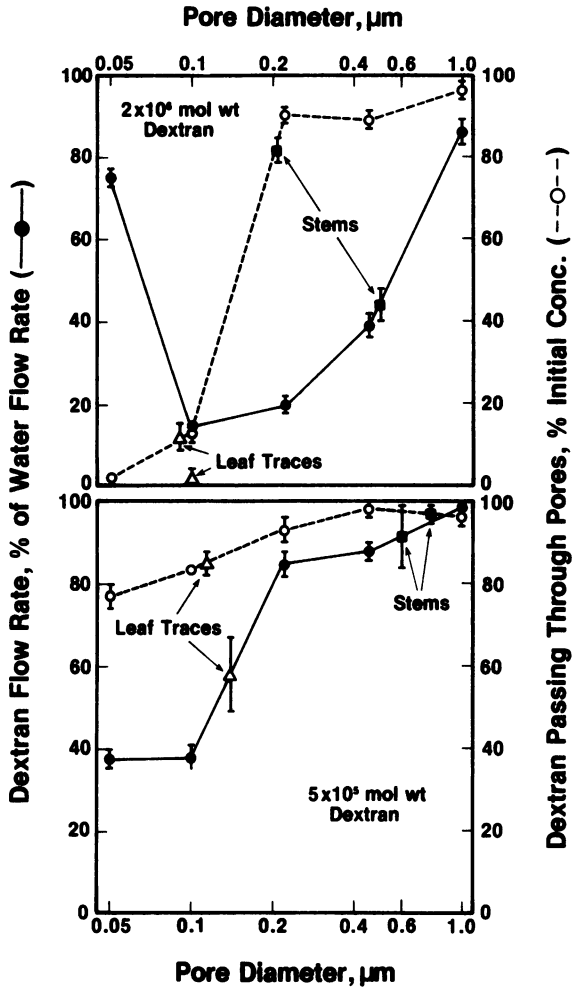

FIG. 1. Flow rate and amounts of dextrans passing through artificial membrane filters as a function of membrane pore size. The same parameters measured for dextrans passing through either alfalfa stems (■) or leaf traces (△) are indicated. For the alfalfa measurements, only the ordinate values are known. Abscissa values are determined from the behavior of the dextrans passing through the membrane filters. Dextrans were at an initial concentration of 0.5 g/l. The flow rate percentage was determined by first measuring the flow rate of water through the filter or vascular tissue and then expressing the flow rate of the dextran solutions as a percentage of this value. Vertical bars represent 2× the SE.

pits, is 0.27 ± 0.2 μm. This value is close to our estimated range of pore sizes of pit membranes in stems. The second type of vessel has helical secondary wall thickenings. The primary wall between the thickenings, which functions as a pit membrane, does not contain visible pores. Possibly, this second type predominates in petiole junctions resulting in smaller effective pore diameters. Generally, vessels with helical thickening are predominant in younger tissue (6).

Autoradiography. Autoradiography was used to determine where a macromolecule in solution accumulates when it is introduced into the xylem. For this study, a macromolecule was used that we felt was too large to pass through any pit membrane pores. The native dextran that is produced by *L. mesenteroides* is a very large molecule that voids gel filtration columns such as Bio Gel A-150 m. We prepared this dextran with a tritium label and introduced it into alfalfa shoots by transpiration. Figure 3 shows the results of this experiment. The silver grains appear to be concentrated where the pits are located, between the helical secondary wall thickenings. Grains were seen only over vessels, indicating that the dextran was unable to pass through the pit

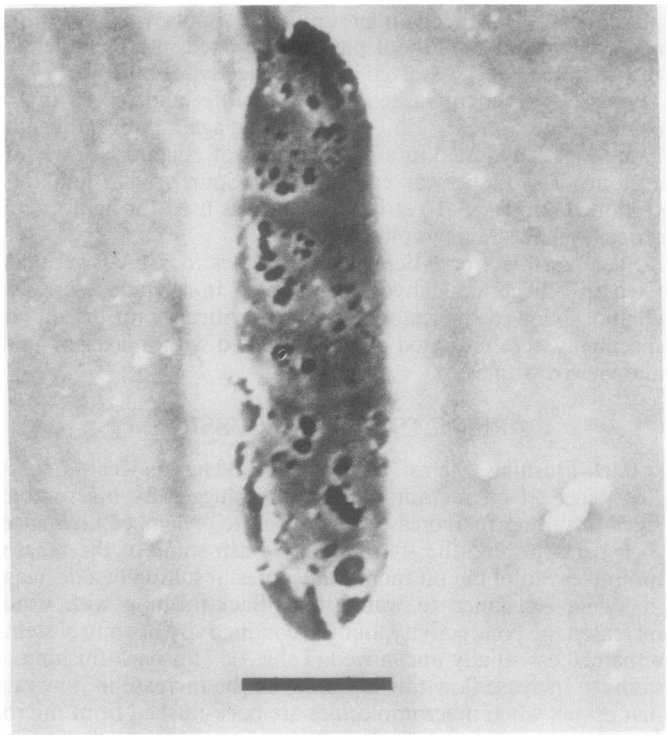

FIG. 2. Scanning electron micrograph of a pit membrane of an alfalfa stem vessel. This micrograph shows the holes present in pit membranes of pitted vessel types. The bar represents 2 μm. Magnification: × 10,000.

membranes. The pit membranes thus filter out the dextran, preventing its movement from vessel to vessel.

Viscosity. Viscosity as a factor in causing the macromolecule-induced increase in xylem resistance plays an insignificant role when the macromolecules are dextrans. The best evidence for this is that xylem resistance did not significantly decrease in the flushing experiment when water followed the dextran treatment on the control stems. If viscosity of the dextran in solution were the factor causing the resistance, once the stems were free of dextran, resistance would decrease to normal levels. This did not occur. It is conceivable that viscosity could play a role in increasing resistance to xylem flow if the macromolecule has a high intrinsic viscosity. That of dextran is relatively low (8).

CONCLUSION

The data presented support the hypothesis that macromolecule-induced reduction of fluid conductivity in vessels is caused by the plugging of pit membranes. Autoradiography shows that very large dextrans accumulate on pit membranes as predicted. Dextrans such as the 2×10^6 D dextran can pass through pit membranes of stems but not through petiole junctions. The 5×10^5 D dextran passes through both sections of the plant's vascular system. Their effects on water movement through the vascular system can be mimicked using membrane filters of known pore size. This use of dextrans of known size was also used to estimate the pore sizes within the pit membranes. The same pores were also measured from SEM micrographs, and these were found to correspond in size to pores measured in stems using the dextran ultra-filtration method. Finally, it was demonstrated that if stems affected by macromolecules are back-flushed, this process restores the ability of stems to conduct water to near normal levels.

If the microfilter model of pit membranes is correct, flow rate through pit membranes of certain pore diameter will be affected only by a fairly narrow size range of macromolecules. In the case of the 2×10^6 D dextran, flow rate is significantly affected by

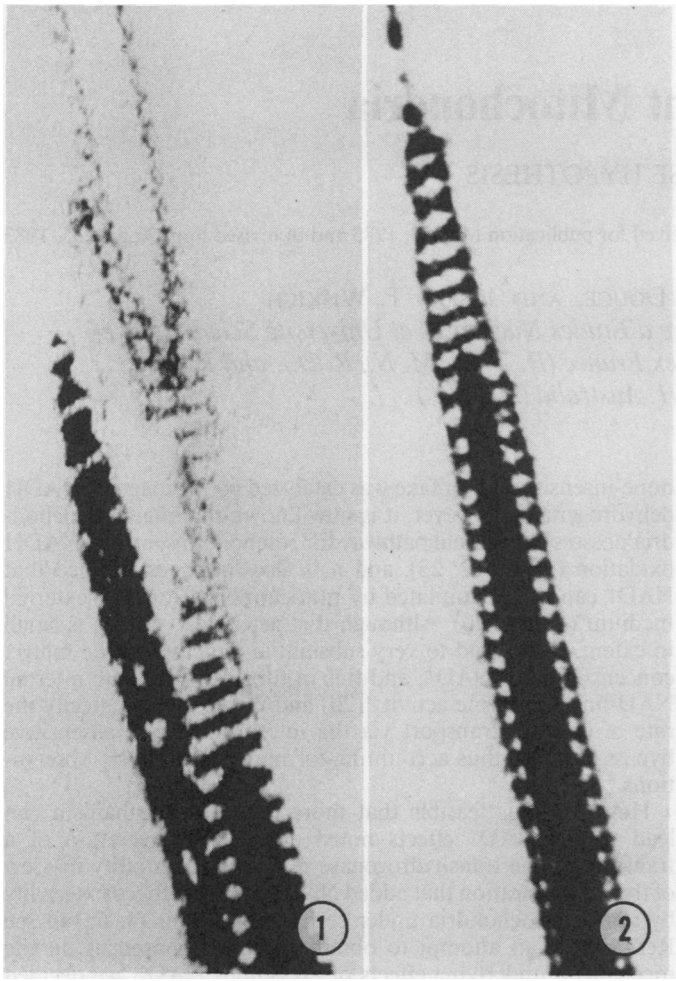

FIG. 3. Autoradiographs showing the location of high mol wt [^{3}H] dextran after being taken up by transpiration into alfalfa shoot cuttings. Autoradiographs were made from unstained longitudinal sections of a stem internode. Magnification: × 525. Two different sections (1 and 2) are shown.

this macromolecule through pores ranging from only 0.1 μm to about 0.6 μm. The narrow pore size range affected by this macromolecule implies that if pathogen produced macromolecules are to interfere with water movement through plants, they must be of a size that corresponds roughly to that of the pore being plugged. A 2×10^6 D molecule would probably be of little pathological significance where pores are greater than 0.6 μm in diameter. From our experience, if this molecule were produced by a pathogen, it would have no significant effect on water movement through stems, but would be capable of totally preventing water movement through leaf traces. The 40 to 50% reduction in stem conductance caused by this molecule is not enough to cause water stress in an alfalfa plant. For instance, we have found that alfalfa stems conduct water in a range of 15 to 50 $\mu l\ s^{-1}\ kPa^{-1}$ (4). At a pressure gradient of 200 KPa m^{-1}, a typical alfalfa shoot can move water through the stem at a rate of 2.2 to 7.2 ml/h. This is at least an order of magnitude greater than the 0.35 g/h transpiration rate that we have measured for such a shoot (4). Thus, a 50% reduction in water flow through the stem would have little effect on transpiration. If our estimate of effective pore size in alfalfa stems is correct, about 90% of the 2×10^6 D molecule would pass through the stem, but it would not be able to pass through the leaf traces, and would completely stop water flow through this part of the xylem.

This hypothetical involvement of a r cromolecule in affecting water flow through alfalfa closely fits the effect the bacterial wilt pathogen *Corynebacterium michiganense* pv *insidiosum* has on alfalfa. We found that this pathogen caused water stress in the plant by essentially stopping water flow through the leaf traces and leaflets but reduced flow through stems by less than 50% (2). This pathogen produces an extracellular polysaccharide that is close to the size of the 2×10^6 D dextran (5×10^6 D). This extracellular polysaccharide component affects water movement in alfalfa much as we have described for the 2×10^6 D molecule (14; N. K. Van Alfen and B. D. McMillan, unpublished). Another macromolecule secreted by this pathogen is approximately the size of the very large dextran used for the autoradiography studies (Fig. 3). This molecule is probably too large to pass through pit membrane pores and would thus remain in the vessel where it is produced until the pathogen enzymically dissolves the pit membrane. A third macromolecule produced by this pathogen is much smaller than the other two (approximately 35,000 D) and would presumedly pass freely through pit membranes but accumulate in the primary walls of leaf cells (N. K. Van Alfen and B. D. McMillan, unpublished). It is possible that movement of water from vessels to evaporative surfaces in the leaves may be affected by this macromolecule (1) and thus contribute to pathogen-induced water stress.

The accumulation of pathogen-produced macromolecules on different sized pores within the water transport capillaries of alfalfa could be responsible for the observed pathogen-caused resistance to water flow. If this is correct, the localization of plant pathogens by pit membranes is not an important defense mechanism in this disease. The pit membranes may in fact contribute to the plant's susceptibility to wilt induced by a pathogen.

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