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Xylem Sap Proteins

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ABSTRACT

Xylem sap from apple (*Malus domestica* Borkh), peach (*Prunus persica* Batsch), and pear (*Pyrus communis* L.) twigs was collected by means of pressure extrusion. This sap contained a number of acidic peroxidases and other proteins. Two other sources of xylem sap used in this study were stem exudates and guttation fluid. Similar peroxidases were also found in stem exudates and guttation fluids of strawberry (*Fragaria* x *ananassa* Duch.), tomato (*Lycopersicum esculentum* L.), and cucumber (*Cucumis sativus* L.). Isoelectric focusing activity gels showed that two peroxidases (isoelectric point [pI] 9 and pI 4.6) were present in initial stem exudates collected in the first 30 minutes after excision. Subsequent samples of stem exudate collected contained only the pI 4.6 isozyme. The pI 4.6 peroxidase isozyme was also found in root tissue and guttation fluid. These observations suggest that roots produce and secrete the pI 4.6 peroxidase into xylem sap. Cucumber seedlings were treated with 100 microliters per liter ethylene for 16 hours and the exudate from decapitated hypocotyl stumps was collected over a 3 hour period. Ethylene increased the peroxidase activity of stem exudates and inhibited the amount of exudate released. These observations suggest that xylem sap peroxidase may play a role in plugging damaged vascular tissue.

The stem exudate of excised watermelon seedlings was shown to contain numerous acidic proteins with a range of mol wt from 10,000 to 100,000 (4). Peroxidase, shikimate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, phosphoglucoisomerase, and phosphoglucomutase were present in this exudate. In subsequent work, stem exudate was shown to contain proteins which inhibited the growth of *Fusarium oxysporum*, the causal agent for *Fusarium* wilt of watermelon (3). The exudate which forms on the stumps of decapitated seedlings is a mixture of phloem and xylem sap. The original purpose of this work was to see if these proteins were located in either phloem or xylem sap.

It was initially thought that these proteins were located in phloem sap. Xylem sap is thought to contain small mol wt inorganic ions and organic compounds and serves as a source of these substances and water to the leaf. However, in 1923 Wilson showed that guttation fluid (essentially xylem sap) from grasses contained enzymes such as catalase, peroxidase, and reductases (10). The possibility that xylem sap contains proteins raises the question of their source, function, and fate. Are xylem sap proteins the result of breakdown products produced during xylem formation or are they produced by parenchyma cells adjacent to xylem tissue? The data presented here suggest that some xylem proteins, such as peroxidase, are specifically secreted into xylem sap by cells located in the root.

Peroxidase isozymes can be used to test the idea that xylem sap contains a unique set of proteins. The presence of a peroxidase isozyme in xylem sap which is not found in the surrounding leaf tissue can be used as evidence that specialized proteins are secreted into the vascular system. Since peroxidases catalyze polymerization reactions, experiments were conducted to test the idea that xylem sap peroxidase plays a role in wound plugging.

MATERIALS AND METHODS

Plant Material

Terminal stems of apple (*Malus domestica* Borkh), peach (*Prunus persica* Batsch), and pear (*Pyrus communis* L.) were collected from the field. Sections of 20 cm by approximately 1 cm were excised from 1-year-old wood. The bark from a 1-cm section of the lower end of the stem was ringed and removed. A plastic hose attached to a syringe was placed over the exposed xylem tissue. Plastic hose clamps were used to minimize leakage. Xylem sap was expressed by forcing a solution of bromophenol blue ($A_{600} = 0.3$) through the tissue. This dye has a negative charge at pH 6. Positively charged dyes such as methyl green adhered to the negatively charged xylem. About 1 mL of exudate was collected by applying pressure to the syringe. Dilution of xylem sap by bromophenol blue forcing solution was calculated by measuring the optical density of the solution collected at 600 nm. Using this technique the respective amount of forcing solution in peach, pear, and apple exudate was 90, 32, and 34%. The SE of these measurements was 5.3%. The difference in xylem sap dilution with forcing solution may be due to the difference in the size of xylem elements. Peach xylem has a larger range of cell diameters than apple and pear. This lack of uniformity would cause most of the forcing solution to bypass smaller cells because large cells are the path of least resistance. Xylem sap was filtered through a 0.22 μm nylon filter prior to concentration on an Amicon Centricon-30 microconcentrator (W. R. Grace, Danvers, MA).

Guttation fluid or stem exudates were collected by means of a handheld pipetter. Guttation fluid was collected from field-grown strawberry (*Fragaria* x *ananassa* Duch.) plants. Guttation fluid was also collected from greenhouse grown tomato plants (*Lycopersicum esculentum* L.). Guttation fluid from tomato was collected after the plants were stored in 100% relative humidity chambers for 18 h. Care was taken

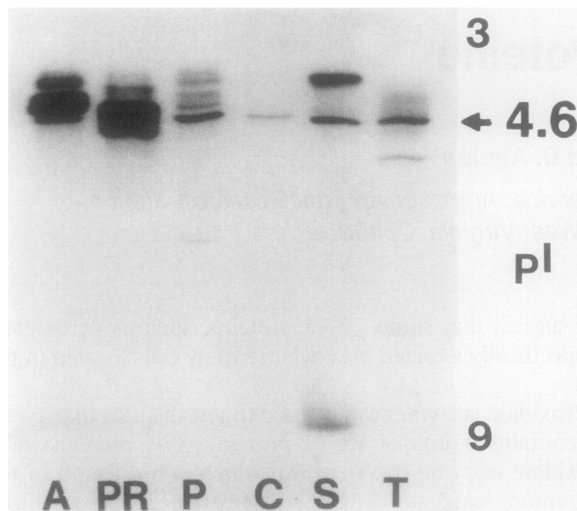

Figure 1. Peroxidase isozymes from apple (A), pear (PR), peach (P), cucumber (C), strawberry (S), and tomato (T) xylem sap. Samples from apple, pear, and peach were extracted by the pressure extrusion method and concentrated 50-fold with an Amicon Centricon-30 microconcentrator. The protein content of the 4 μL sample was 1.2 μg for apple and 0.4 μg for pear and peach. The cucumber guttation fluid was not concentrated prior to electrophoresis. The 4 μL sample contained 0.012 μg protein. The strawberry and tomato guttation fluids were concentrated 50-fold and contained 0.4 μg protein. The arrow indicates the pI 4.6 peroxidase.

not to disrupt glandular hairs on the leaf surface. Cucumber seedlings (*Cucumis sativus* L. "Straight Eight") were grown and treated with 100 μL/L ethylene or air for 18 h in sealed chambers (2).

Guttation fluid was collected from cotyledon and leaf margins. Plants were decapitated below the cotyledon and the stem exudate collected after the cut surface was washed with water and blotted dry with a paper towel. Samples were collected every 30 min over a 3 h period. Samples were stored at −20°C until used. There were three replications per treatment. Each replication consisted of three pots with five plants per pot.

Proteins were also extracted from acetone powders of roots, stems, cotyledons, and leaves of cucumber seedlings. Tissue (2 g) was homogenized in 4 mL of 70% acetone. The homogenate was filtered through Whatman No. 1 paper on a filter funnel and washed with 100% acetone. After air drying, proteins were extracted with 1 mL of 0.1 M (pH 6.8) KPO_4 in a 1.5 mL Eppendorf centrifuge tube. After centrifugation for 2 min at 15,000*g*, the supernatant was used in enzyme assays (1).

Electrophoresis

SDS-PAGE was run using 10 to 15% acrylamide gradient gels from Pharmacia (Piscataway, NJ) on the Pharmacia Phastsystem. The electrophoretic and staining techniques were those recommended by Pharmacia. IEF[1]-PAGE was run using Pharmacia gels with ampholytes in the 3 through 9 or 4 through 6.5 range as described previously (2). Silver staining was used to visualize proteins in both separation systems. The method used to silver stain gels and the mol wt and IEF standards were obtained from Pharmacia. Peroxidase activity in gels was visualized with a 10 mM guaiacol/10 mM H_2O_2 substrate.

Spectrophotometric Analysis

Protein concentration was determined by the Bio-Rad protein dye assay following the directions supplied by Bio-Rad.

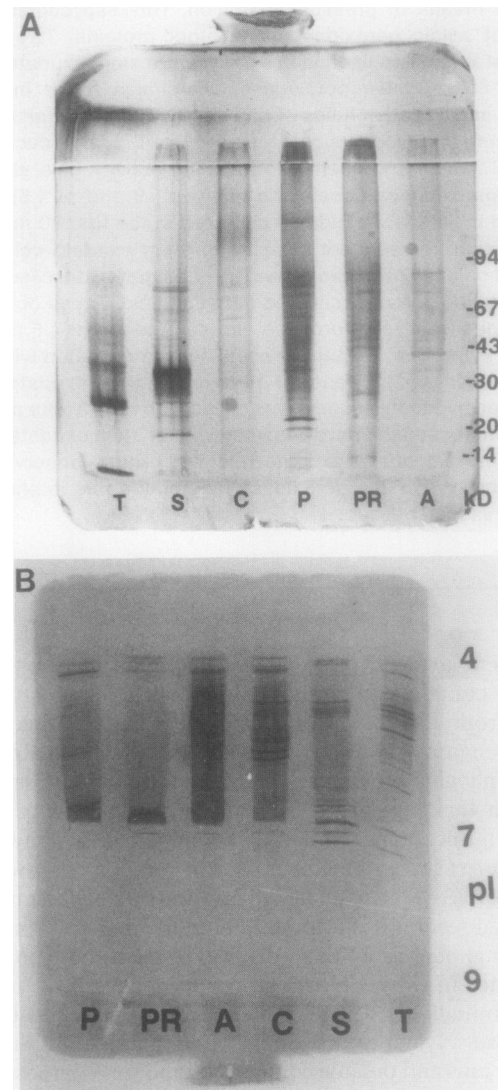

Figure 2. Xylem proteins from apple (A), pear (PR), peach (P), cucumber (C), strawberry (S), and tomato (T) xylem sap. The proteins used in these samples were similar to those used above except that the cucumber guttation fluid was also concentrated 50-fold and the 4 μL sample had a protein content of 0.6 μg protein. A, The size range of xylem sap proteins. The mol wts of the protein standards are shown in the right lane. B, The charge of these proteins. The pI of protein standards is indicated in the right lane.

[1] Abbreviations: IEF, isoelectric focusing, pI, isoelectric point.

The specific activity of peroxidase was measured with 1 mM guaiacol and 1 mM H_2O_2 as substrates and expressed as the change in absorbance at 470 nm/min·mg of protein.

RESULTS

IEF electrophoresis of xylem sap proteins from woody and herbaceous plants indicated the presence of a number of acidic peroxidases (Fig. 1). All contained a peroxidase with a pI of 4.6. Four peroxidase isozymes were evident in the xylem exudate of apple (A), pear (PR), and peach (P). One isozyme with a pI of 4.6 was observed in cucumber (C) and three isozymes were observed in strawberry (S) and tomato (T). Not seen in this figure is a cucumber guttation peroxidase with a pI of 4. This peroxidase was evident when a more concentrated sample was applied to the gel (as will be shown later). Unlike other plants, strawberry had a basic peroxidase in its guttation exudate. Specific peroxidase activity (±SE) of peach, apple, and pear exudate was 0.925 ± 0.525, 10 ± 0.83, and 5.6 ± 0.525 in one experiment with 4 to 5 replications per treatment.

The mol wt and charge diversity of proteins present in the above xylem sap samples is shown on SDS-PAGE (Fig. 2A) and IEF (Fig. 2B) gels. The same amount of xylem sap was collected from each deciduous tree stem, filtered with a 0.22 μm filter, and then concentrated 50-fold with a microconcentrator. Apple xylem sap contained the highest level of protein with 300 μg/mL, whereas peach and pear xylem sap contained approximately 100 μg/mL. After concentration, the cucumber, strawberry, and tomato samples contained 150, 100, and 100 μg/mL of protein, respectively. The size of proteins present in xylem sap ranged from 10 to 100 kD. IEF gel electrophoresis showed that the xylem sap proteins were primarily negatively charged and had pIs from 4 to 7.

The effect of ethylene and time after excision on cucumber stem exudate peroxidase activity is shown in Figure 3. The protein content of the exudate decreased about 90% after the first 30 min collection period. The protein content of subsequent stem exudates was relatively constant. However, total peroxidase activity of the stem exudate remained constant over the 3 h collection period. The data in Figure 3 indicate that the peroxidase activity of stem exudate from ethylene-treated (100 μL/L for 16 h) plants was greater than from the control. Ethylene also increased the peroxidase activity of guttation fluid by threefold. After 16 h the specific activity of cotyledon and leaf guttation fluid peroxidase from ethylene-treated seedlings was 2.19 compared with 0.67 for controls. Five samples were used to determine these values and the LSD = 0.44.

Protein samples from control (A) and ethylene-treated (E) plants from the first (A1, E1) and last (A4, E4) 30 min collection period were separated on IEF and SDS-PAGE gels. A sample of ethylene-treated cotyledon protein (C) was used to show the total number of isozymes observed in predominately nonvascular tissue. As shown in an IEF gel (Fig. 4A), ethylene increased peroxidase activity (compare A1 to E1).

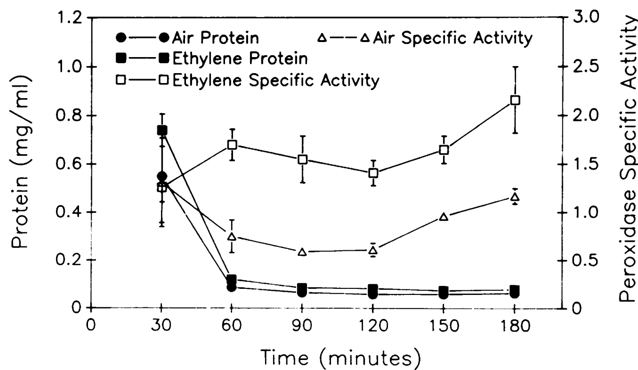

Figure 3. The effect of ethylene on xylem sap peroxidase activity from cucumber stems. Points represent total protein and peroxidase activity collected at 30 min intervals. Error bars indicate the SE.

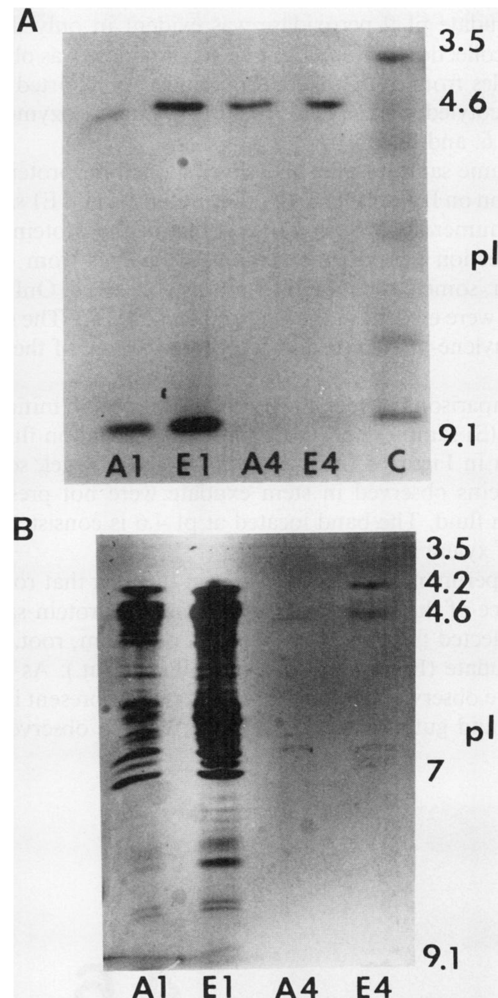

Figure 4. A, Xylem exudate peroxidase isozymes from cucumber seedlings. Peroxidase activity was visualized with guaiacol/H_2O_2. Samples labeled A1 (air) and E1 (100 μL/L ethylene) were collected during the first 30 min. Samples labeled A4 and E4 were collected between 3.5 and 4 h from air and ethylene-treated seedlings, respectively. Lane C contains the peroxidase isoenzymes from ethylene-treated (16 h) cotyledons. All xylem exudate samples were concentrated 40-fold. B, Xylem exudate proteins from ethylene-treated cucumber seedlings separated on an IEF gel. See A for details.

Stem exudate pI 9 peroxidase was evident in only the first sample collection period. The pI 4.6 peroxidase was observed in samples from both collection periods. As reported earlier (1), the cotyledon sample also had peroxidase isozymes with pIs at 9, 6, and 4.

The same samples were also silver stained for protein after separation on IEF gels (Fig. 4B). The initial A1 and E1 samples exhibit numerous protein bands. Most of the proteins from this collection period were acidic and had pIs from 4 to 7. However, some basic proteins were also observed. Only a few proteins were evident in the 4 h samples (A4, E4). The sample from ethylene-treated seedlings contained more of the pI 4.6 protein.

A comparison between the proteins observed in initial stem exudate (S) samples and those present in guttation fluid (G) is shown in Figure 5. In this silver stained IEF gel, some of the proteins observed in stem exudate were not present in guttation fluid. The band located at pI 4.6 is consistent with the pI of xylem sap peroxidase.

An experiment was designed to test the idea that roots are the source of the pI 4.6 peroxidase (Fig. 6). Protein samples were collected from leaf, cotyledon (Cot.), stem, root, initial stem exudate (Exud.) and guttation fluid (Gut.). As shown above, we observed that pI 4.6 peroxidase was present in stem exudate and guttation fluid samples. We also observed that this protein was present in peroxidases isolated from roots. The pI 4.6 peroxidase was not seen in samples isolated from leaf, cotyledon, or stem tissue. A pI 4 peroxidase was also seen in guttation fluid.

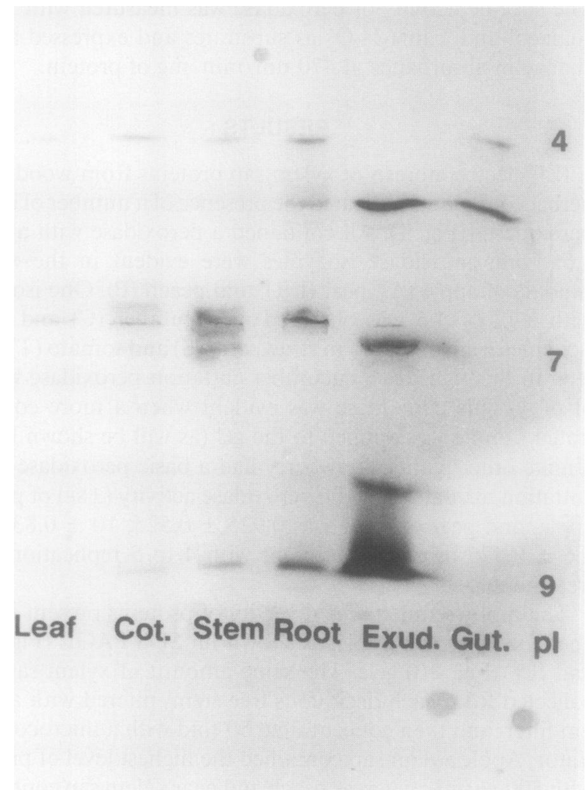

Figure 6. Protein samples were collected from leaf, cotyledon (Cot.), stem, root, initial stem exudate (Exud.), and guttation fluid (Gut.). Stem, root, stem exudate, and guttation fluid samples were concentrated 10-fold with an Amicon Centricon-30 filter.

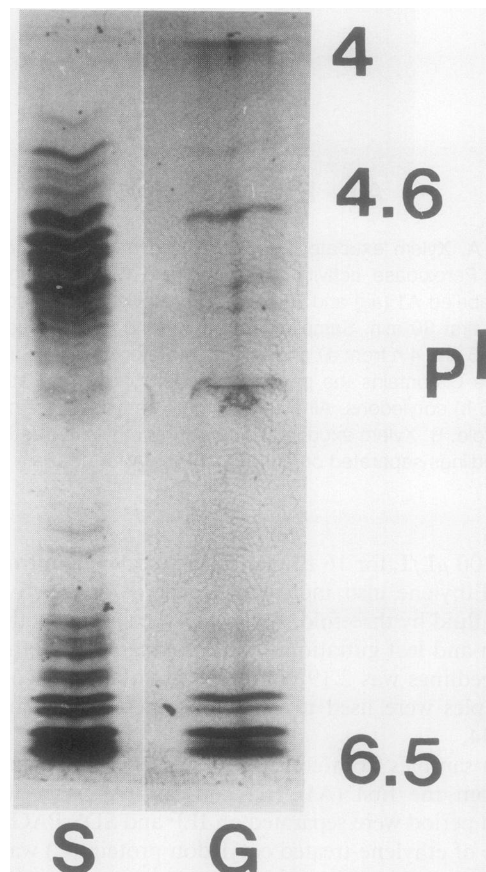

Figure 5. Comparison between total xylem proteins from stem exudate (S) and guttation fluids (G) from control cucumber plants.

Decapitation of the cucumber seedling results in the presence of stem exudate on the cut surface. With time, the flow of this exudate is reduced. As shown in Figure 7, pretreating the seedlings with ethylene reduced xylem sap flow.

DISCUSSION

The data shown in Figure 1 indicate that xylem sap from fruit trees and other plants contains a number of acidic peroxidases. In addition, the data shown in Figure 2B indicate that most of the proteins present in xylem sap also have a negative charge. The negative charge of these proteins may facilitate their passage through xylem cells. Xylem cell walls are thought to have a negative charge because positively charged dyes such as methyl green are absorbed from solutions forced through vascular tissue. The bromophenol blue used here has a negative charge and was not absorbed by stem tissue.

The stem exudate collected from decapitated seedlings during the first 30 min had a large amount of protein. With time, the total amount of protein in the exudate decreased but the levels of peroxidase remained nearly constant (see Fig. 3). The

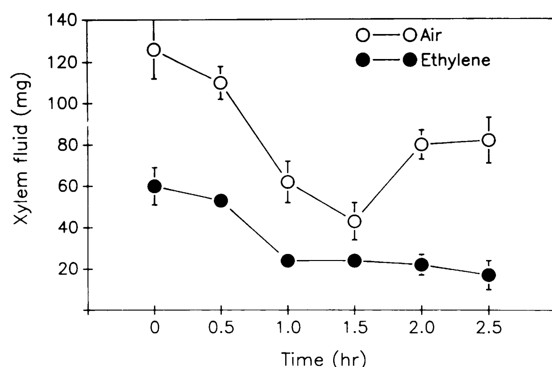

Figure 7. The effect of ethylene on the amount of stem exudate production by cucumber stems.

electrophoretic studies shown in Figure 4A and B indicated that only a few proteins were present in subsequent collections of stem exudate. Since both phloem and xylem are cut after decapitation of the seedling, initial stem exudate proteins are a mixture of phloem and xylem sap. Subsequent samples are presumed to have a larger amount of xylem sap proteins.

Guttation fluid accumulating at the margin of leaves under the conditions of high humidity is primarily xylem sap. After xylem sap leaves the vascular system it passes through a layer of parenchyma cells between the end of the vascular strands and the hydathode. Some of the proteins seen in guttation fluid may have been washed out of the intercellular spaces of these cells. Figure 5 shows that a larger number of proteins were present in stem exudate (S) than in guttation fluid (G). However, both samples contained proteins with a pI of 4.6. As shown in Figure 6, a pI 4.6 peroxidase was present in both stem exudate and guttation fluid.

As shown in Figure 6, we observed that the pI 4.6 peroxidase was present in root extracts. We did not observe this isozyme after loading similar amounts of total peroxidase activity from leaves, cotyledons, and stems on the gel. The pI 4.6 isozyme reappeared in both stem exudate and guttation fluid. These observations are consistent with the idea that roots are the source of this peroxidase. The role of the pI 4.6 and pI 4 peroxidase in xylem sap is not known. Proteins may occur in xylem sap as the indirect result of trachied development. They may also be secreted or leak from adjacent parenchyma cells. If peroxidase and other proteins are actively secreted into xylem sap, then the question of the role and fate of these proteins must be answered.

As shown in Figure 3, ethylene increased the levels of xylem sap peroxidase. This is consistent with earlier work showing that ethylene increased the levels of pI 4 and pI 9 peroxidase in cotyledonary tissue (1). Plants have a number of peroxidase isozymes. The role of these proteins is still unknown (8). However, there is general agreement that peroxidases catalyze the polymerization of *para*-hydroxycinnamyl alcohols, long chain fatty acids, hydroxylated fatty acids, and long chain alcohols into lignin and suberin. In cucumber, as in other plants, wounds are sealed off by a polymer or plug formed on cut surfaces (9). The presence of peroxidase in these exudates suggests that it may be involved in the formation of these plugs. The monomers which produce these clots were not characterized. These wound plugs are probably involved in preventing desiccation, preventing infection by microorganisms, and facilitating the formation of wound periderm (9). Recently, Lagrimini *et al.* (5) reported that transformed tobacco plants, which overproduced an acidic peroxidase, wilted at the time of flowering. Ethylene-induced gel formation occurs in castor bean (7) and sour cherry (6) stems. These observations also suggest that acidic peroxidases are involved in vascular plugging.

In conclusion, we have observed that xylem sap contains acidic peroxidases and other proteins. The data presented in Figure 6. are consistent with the idea that roots may be the source of these proteins. The pI 4.6 peroxidase found in cucumber xylem sap was found in low amounts in leaf tissue. This suggests that some mechanism exists to secrete specific proteins into this vascular fluid.

Transpiration would result in the accumulation of xylem sap proteins in the leaf. In the case of some herbaceous plants, these proteins would be disposed of by guttation. The means by which woody plants remove and dispose of these proteins from the transpiration stream is unknown.

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