

## Mechanism of Wilt Induction by Amylovorin in Cotoneaster Shoots and Its Relation to Wilting of Shoots Infected by *Erwinia amylovora*

Thomas M. Sjulin and Steven V. Beer

Former Liberty Hyde Bailey Research Assistant and Associate Professor, respectively, Department of Plant Pathology, Cornell University, Ithaca, NY 14853.

Present address of senior author: Department of Plant Pathology, University of Illinois, Urbana, IL 61801.

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### ABSTRACT

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The effects of amylovorin, a wilt-inducing polysaccharide isolated from fire blight ooze, on the water relations and cell permeability of *Cotoneaster pannosa* shoots were not related to changes observed in shoots infected by *Erwinia amylovora*. Rates of solution uptake and transpiration by shoots in amylovorin solution were less than in water. Water potentials and water conductance of stem segments were reduced significantly in amylovorin-wilted shoots relative to non-wilted shoots. The effect of amylovorin solutions on water potentials could be reproduced by dextran solutions or by sealing shoot bases with wax. Electrolyte loss from wilted tissues of shoots exposed to amylovorin was no different than loss from nonwilted tissues of shoots held in water. In contrast, water potentials of infected shoots were significantly

higher than those of noninoculated shoots. Water potentials of apparently healthy tissues distal from infected tissues in shoots inoculated 7 cm behind the shoot apex were not significantly different from comparable tissues of non-inoculated shoots. Electrolytes were lost at a much higher rate from wilted tissues of infected shoots than from healthy tissues of noninoculated shoots. We conclude that amylovorin induces wilt by restriction of water movement in xylem, rather than by a directly toxic effect as previously suggested. However, the changes in water relations of shoots infected by *E. amylovora* suggest that wilt of such shoots results from a disruption of membrane integrity, rather than by restriction of water movement.

*Erwinia amylovora* (Burrill) Winslow et al., causes rapid blighting of blossoms and vegetative shoots of susceptible plants. The first symptoms of the disease (fire blight) are water-soaking and wilting of affected tissues; necrosis follows. A sticky exudate or ooze containing bacteria often is associated with infected tissues (8).

Previous workers have reported that bacteria-free preparations from ooze or *E. amylovora*-infected tissues induce wilting of detached shoots (9, 10, 11, 17). Goodman et al. (10) isolated from ooze a high-molecular-weight polysaccharide, amylovorin, which was characterized as a host-specific toxin. When shoots of rosaceous plants were placed in dilute amylovorin solution, they wilted in a period of time that was negatively correlated with their susceptibility to *E. amylovora* in the field. Shoots of six nonrosaceous species tested did not wilt in amylovorin solutions. Based on observations of increased uptake of Evans' blue dye by cells of amylovorin-treated shoots, Huang and Goodman (12) suggested that wilting induced by amylovorin solutions resulted from increased permeability of membranes to solutes.

Polysaccharides and glycopeptides induce wilting by either disrupting membrane semipermeability or physically restricting water movement (6). The purposes of the present studies were to determine the mechanism by which amylovorin induces wilting in detached shoots, and to relate this mechanism to the water relations of

*E. amylovora*-infected shoots. Preliminary reports of this work have been published (22, 23).

### MATERIALS AND METHODS

**Production and isolation of amylovorin.** — Amylovorin, which was used in all experiments unless stated otherwise, was isolated from ooze produced on immature (2- to 3-cm diameter) pear fruits (*Pyrus communis* 'Bartlett') inoculated with *E. amylovora* isolate 27-3 (20). The procedures of Goodman et al. (10) were used for isolation and purification of amylovorin, with the following modifications. Ooze was collected and diluted with 1.0 M sodium chloride, instead of water, to enhance the subsequent precipitation of polysaccharide with ethanol. The polysaccharide solution was dialyzed against 20 volumes of water (changed once) at 4 C for 48 hr prior to lyophilization.

**Comparison of amylovorin preparations.** — Comparative analyses of the polysaccharide isolated from infected pear fruits and of authentic amylovorin [isolated from infected apple (*Malus pumila* 'Jonathan') fruits and kindly provided by R. N. Goodman] were made to determine if the two preparations were similar.

Polysaccharide samples in 1 mM Na<sub>2</sub>EDTA (pH 7.0) were applied to 2.5 × 40 cm columns of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden). Samples were eluted by gravity flow with 1 mM Na<sub>2</sub>EDTA (pH 7.0) at approximately 0.5 ml/min. Fractions were collected at 2-min intervals and assayed for carbohydrate by the anthrone reaction (25). Column void volumes were

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determined with Blue Dextran 2000 (Pharmacia Fine Chemicals). Elution peaks of the infected pear polysaccharide were tested for biological activity. Fractions with anthrone activity were pooled, dialyzed against 100 volumes of water (changed once) for 24 hr at 4 C, and lyophilized. Solutions of the recovered polysaccharide (100  $\mu\text{g}/\text{ml}$ ) were compared with 100  $\mu\text{g}/\text{ml}$  solutions of polysaccharide not subjected to gel filtration for the ability to wilt shoots as described below.

The polysaccharides also were compared in compositional studies to be published elsewhere (Beer et al., unpublished). Preparations of both polysaccharides were subjected to methanolysis and the trimethylsilyl derivatives of the resulting products were separated and estimated quantitatively by gas-liquid chromatography. An antiserum prepared in rabbits against the polysaccharide from pear ooze was used to compare both polysaccharides in immunodiffusion assays.

**Plant material.** — *Cotoneaster pannosa* Franch. was used in all experiments because of its susceptibility to *E. amylovora* (24) and the ease with which uniform vegetative shoots could be produced in a greenhouse. Plants were grown from rooted cuttings in a peat: vermiculite mix (1:1, v/v) at  $24 \pm 3$  C with a photoperiod of at least 14 hr and sufficient water and fertilizer to maintain vegetative shoot extension.

Pairs of visually uniform shoots on individual plants were selected for inoculation to obtain infected plant material. One shoot of each pair was inoculated with a suspension of *E. amylovora* (isolate 27-3) from a 24-hr nutrient agar slant culture. Bacteria were introduced into shoot apex or stem tissues with a No. 3 insect mounting pin. The other shoot was wounded with a sterile pin in a similar fashion.

#### Assays of wilt induction by polysaccharide solutions.

— Solutions of amylovorin purified from infected apple and pear fruits were tested for their ability to induce wilt of detached shoots. Solutions of T-500 Dextran and Blue Dextran 2000 (Pharmacia Fine Chemicals) were also tested, since amylovorin eluted between these two polysaccharides from columns of Sepharose 6B gels (21). Visually uniform shoots (25-50 cm long) were removed from plants, cut to desired length under water, and allowed to equilibrate in distilled water under assay conditions ( $23 \pm 1$  C, 70% relative humidity and 17 klux) for at least 1 hr before assays were begun. Shoots were each transferred into 2-ml amounts of test solution in a 5-ml vial; at least six shoots were tested for each solution. Shoots were designated as wilted when the apex bent 90 degrees from normal.

**Measurements of rates of water uptake and transpiration.** — Transpiration rates were estimated by weighing shoots plus their individual vials ( $\pm 10$  mg) at 40-min intervals. Water uptake rates were estimated also at 40-min intervals by weighing vials after removal of shoots. Evaporative losses from vials without shoots were negligible throughout the experiment.

**Water potential determinations.** — Water potentials of shoots were determined by the pressure chamber method (2, 19). Apical 5-cm portions were removed from shoots and placed immediately into a pressure chamber (Model 1000, PMS Instrument Co., Corvallis, OR 97330) such that the lower 2-cm portion (with leaves removed)

extended through rubber seals to the outside of the chamber. The water potentials of *E. amylovora*-infected shoots were determined 3 days after they had been inoculated 0.5 cm from the apex.

**Water conductance in stem segments.** — Water conductance by stem segments was measured by a method similar to that used by Van Alfen and Turner (26). Immediately after a water potential determination, the 10-cm segment located 5 to 15 cm behind the apex was removed under water and placed in the pressure chamber with the basal end immersed in water which had been membrane-filtered (0.45- $\mu\text{m}$  pore size). The apical end protruded from the chamber and was connected to a horizontal 1-ml pipette (graduated in 10- $\mu\text{l}$  divisions) with a short length of tubing filled with water. Pressure within the chamber quickly was increased to 2.07 bars, and the time required to pass 10  $\mu\text{l}$  of water through the segment was recorded after a constant rate was achieved.

**Cell permeability measurements.** — The effect of amylovorin solutions on cell permeability was estimated by measuring electrolyte loss from leaf disks (4). *Cotoneaster* leaf disks, 8 mm in diameter (0.3 g fresh wt), were bathed for 6 hr in 30 ml of 0.1 mg/ml amylovorin, 1.0 mg/ml amylovorin, or deionized water at  $25 \pm 0.5$  C on a rotary (180 rpm) platform shaker. After a quick rinse with deionized water, disks were transferred to 30-ml portions of deionized water and shaking was continued. The electrical conductance of the bathing solution was measured with a conductivity bridge and cell (Models 31 and 3403, respectively, Yellow Springs Instrument Co., Yellow Springs, OH 45387) at 30-min intervals. Electrolytes that remained in the tissues after bathing in water for 3.5 hr were liberated by freezing and thawing. Shaking and conductance measurements then were resumed until a constant reading was obtained.

The permeability of stem tissues that had been wilted by amylovorin or by infection with *E. amylovora* was estimated similarly. Four 0.5-cm segments located 1.0 to 3.0 cm behind the shoot apex were removed and placed in 4 ml of deionized water on a wrist-action shaker at  $23 \pm 1$  C. Electrical conductance of the bathing solution was measured at 30-min intervals for the next 4 hr. Electrolytes remaining in the tissues were estimated as described above.

## RESULTS

**Comparison of amylovorin preparations.** — The polysaccharide isolated from infected pear fruits was eluted from gel filtration columns as a single peak which coincided with the peak of authentic amylovorin (Fig. 1). This elution peak contained  $99 \pm 5\%$  of the total anthrone-positive material that had been applied to the column. Solutions of the polysaccharide from pear ooze (100  $\mu\text{g}/\text{ml}$ ) recovered from the column induced wilting of 10-cm-long *cotoneaster* shoots in 95 min, whereas 100  $\mu\text{g}/\text{ml}$  solutions not subjected to gel filtration induced wilting in 142 min (no significant difference,  $P=0.05$ ).

Compositional studies (to be reported in detail elsewhere) indicated that the methanolysis products of authentic amylovorin and the polysaccharide isolated from pear ooze did not differ. The chromatograms from both preparations had seven major peaks. The retention

times of each peak, relative to an internal standard, were similar for both preparations. The shape and corrected area of each of the seven peaks from authentic amylovorin and from the polysaccharide from pear ooze were similar. These results indicated that the two preparations contain the same components and in the same relative proportion. Galactose, glucose, and glucuronic acid were identified in both preparations by cochromatography of the treated polysaccharides with authentic sugars and uronic acids. Authentic amylovorin reacted with antiserum produced against the polysaccharide from pear ooze in a manner similar to that of the homologous combination; no spurs formed when the polysaccharide from pear ooze was placed in wells adjacent to those that held authentic amylovorin in microslide double-diffusion assays in agar gels.

**Appearance of amylovorin-wilted shoots and shoots infected with *Erwinia amylovora*.** — Wilting induced by amylovorin solutions in cotoneaster shoots was visibly expressed as a loss of turgor by the succulent tissues of the stem. Although it was not visibly expressed, loss of leaf turgor (as determined by touching the leaves) apparently occurred in amylovorin-wilted shoots. Wilting induced by amylovorin solutions was indistinguishable from wilting induced by dextran solutions or by sealing shoot

bases with wax (Tissuemat, melting point 52.5 C, Fisher Scientific, Rochester, NY 14624).

Wilting induced by amylovorin solutions was similar, but not identical, to wilting observed in shoots infected with *E. amylovora*. Wilting of shoots inoculated with *E. amylovora* 0.5 cm from the apex was restricted to the succulent tissues of the stem. Loss of turgor and internal water-soaking of apical stem tissues, but not of leaf tissue, were apparent in all shoots 3 days after inoculation. When shoots were inoculated with *E. amylovora* 7 cm from the apex, no wilting was observed in tissues distal to the point of inoculation after 5 days, despite the appearance of bacterial exudate at several locations on the stem and, in some cases, partial collapse of the stem at the point of inoculation.

**Effect of amylovorin on shoot water relations.** — Rates of solution uptake and transpiration by shoots decreased in amylovorin solutions relative to rates in water (Fig. 2). The mean rate of solution uptake by shoots placed in 1.0 mg/ml amylovorin solutions was significantly lower ( $P = 0.05$ ) after 80 min of treatment than that of shoots that remained in water. Transpiration rates of amylovorin-treated shoots were less than water-treated control shoots after 120 min; a significant difference ( $P = 0.01$ ) was noted after 160 min. Shoots in amylovorin solutions wilted in an average of 168 min during this assay.

The wilt-inducing ability and effect on shoot water potentials of 100  $\mu\text{g/ml}$  amylovorin solutions were compared to those of 100  $\mu\text{g/ml}$  solutions of dextrans of similar size (Table 1). There were no significant differences in the wilt-inducing ability of solutions of amylovorin prepared from ooze from infected apple or pear fruits or of Dextran T-500. Water potentials of shoots wilted in all polysaccharide solutions tested were significantly more negative ( $P = 0.01$ ) than the water potentials of nonwilted control shoots, but were not

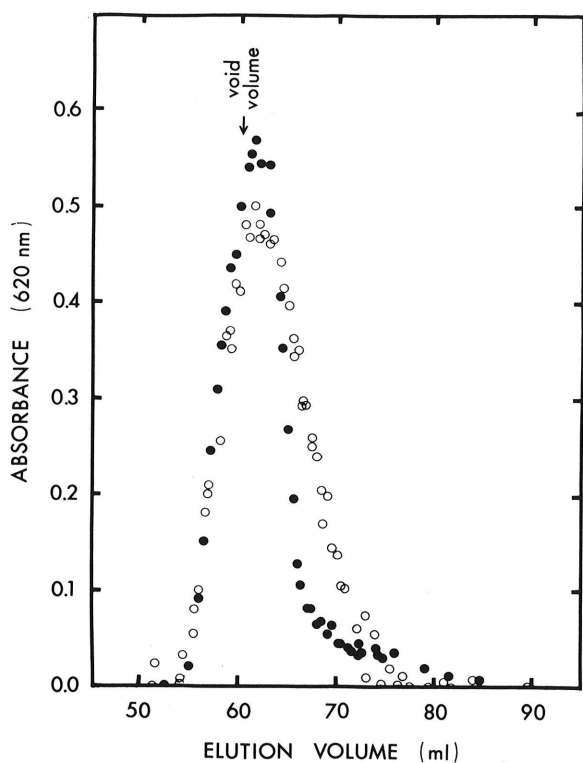


Fig. 1. Elution profiles of authentic amylovorin (closed circles) and the polysaccharide isolated from *Erwinia amylovora*-infected *Pyrus communis* 'Bartlett' fruits (open circles). Preparations (1.0 mg) were applied to a 2.5 × 40-cm column of Sepharose 6B and eluted with 1 mM Na<sub>2</sub>EDTA (pH 7.0). Column void volume was determined with Blue Dextran 2000. Polysaccharide content of fractions was determined by the anthrone reaction.

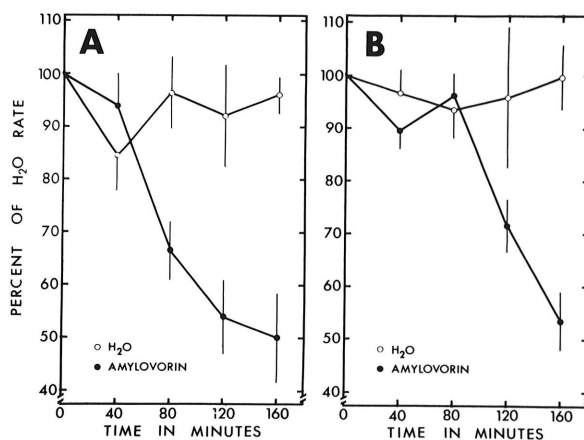


Fig. 2.—(A, B). Rates of A) water uptake and B) transpiration by 10-cm *Cotoneaster pannosa* shoots in water or 1.0 mg/ml amylovorin solution. Rates are expressed as a percent of the mean rate of each shoot measured in water for the 3-hr period before the start of the assay. Data points represent the mean rate for the previous 40 min for four shoots in water and sixteen shoots in amylovorin; vertical lines depict the standard error of the means.

TABLE 1. Effect of amylovorin and dextran solutions, and wax on time to wilt and water potentials of *Cotoneaster pannosa* shoots<sup>a</sup>.

Treatment	Time to wilt (hr)	Water potential (bars)
Water <sup>b</sup>	...	-8.6 A
Wax	0.35 A	-15.6 B
Amylovorin <sup>c</sup>	1.06 A B	-15.6 B
Amylovorin <sup>d</sup>	1.85 B	-15.5 B
Dextran T-500	1.67 B	-15.7 B
Blue Dextran 2000	3.36 C	-15.3 B

<sup>a</sup>Cotoneaster shoots (10-cm) were placed in water or 100  $\mu\text{g/ml}$  polysaccharide solutions or their bases were sealed with wax. Shoots were held at  $23 \pm 1\text{C}$ , 70% relative humidity and 17 klux. At the time of wilt (when the shoot apices bent 90 degrees from normal) water potentials were determined by the pressure chamber method. Values in a column not followed by the same letter were significantly different ( $P = 0.05$ ) according to Duncan's new multiple range test.

<sup>b</sup>One of eight shoots in water wilted during the assay; the water potentials of the seven nonwilted shoots were determined after 3.0 to 3.5 hr.

<sup>c</sup>Isolated from ooze produced on *Erwinia amylovora*-infected *Malus pumila* 'Jonathan' fruits by R. N. Goodman.

<sup>d</sup>Isolated from ooze produced on *E. amylovora*-infected *Pyrus communis* 'Bartlett' fruits.

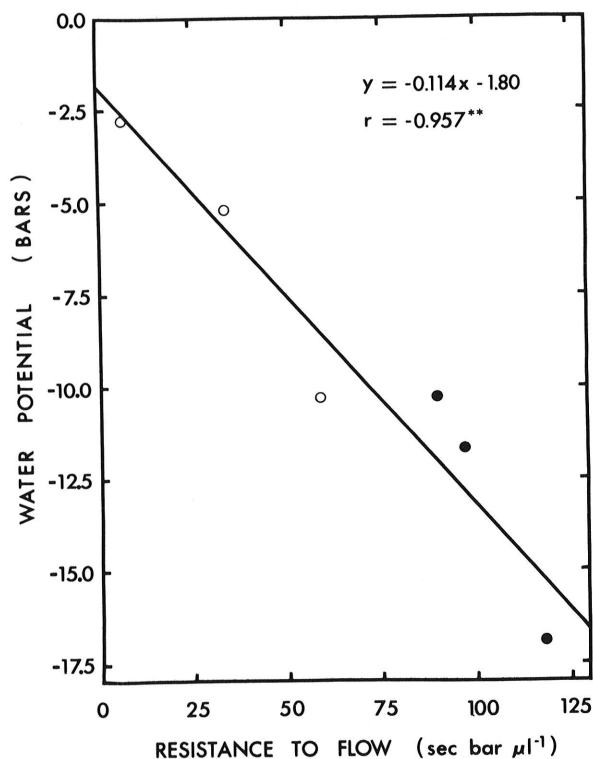


Fig. 3. Water potentials of the apical 5-cm portions of 20-cm *Cotoneaster pannosa* shoots as a function of the resistance to flow of stem segments located 5-15 cm behind shoot apices. Open circles represent data from nonwilted shoots in water and closed circles represent data from wilted shoots in amylovorin solutions (100  $\mu\text{g/ml}$ ).

different from water potentials of shoots wilted by sealing their bases with wax.

The water conductance rate of stem segments from amylovorin-wilted shoots ( $9.8 \mu\text{liters sec}^{-1} \text{bar}^{-1}$ ) was significantly lower ( $P = 0.05$ ) than the conductance rate of segments from water-treated control shoots ( $29.9 \mu\text{liters sec}^{-1} \text{bar}^{-1}$ ). Furthermore, there was a highly significant ( $P = 0.01$ ) and negative correlation between the resistance to water flow through the stem segments (conductance<sup>-1</sup>) and the water potential of the apical tissues of the same shoots (Fig. 3).

**Effect of amylovorin on cell permeability.** — After exposure of cotoneaster leaf disks to amylovorin solutions for 6 hr, no alteration in their ability to retain solutes could be detected. No significant differences in electrolytes remaining in tissues were observed among leaf disks exposed to water ( $114 \pm 2.8 \mu\text{mho}$ ), 0.1 mg/ml amylovorin solutions ( $113 \pm 5.7 \mu\text{mho}$ ) or 1.0 mg/ml amylovorin solutions ( $114 \pm 3.0 \mu\text{mho}$ ).

The permeability of stem tissues wilted by amylovorin solutions was compared to that of stem tissues in water-treated shoots (Fig. 4-A). No difference in electrolyte loss was detected between stem segments from the wilted region of amylovorin-treated shoots and segments from comparable positions on water-treated shoots.

#### Water relations of *Erwinia amylovora*-infected shoots.

— The mean water potential of infected shoots ( $-2.2$  bars) was significantly less negative ( $P = 0.01$ ) than the mean water potential of noninoculated shoots ( $-4.1$  bars) in a sample of 10 pairs. Water potentials also were determined in shoots 5 days after inoculation at a point 7 cm behind the apex. The mean water potential of apparently healthy tissues distal to infected tissues ( $-2.8$  bars) was not significantly different ( $P = 0.05$ ) from that of non-inoculated controls ( $-3.2$  bars) in a sample of five pairs.

**Permeability of tissues infected with *Erwinia amylovora*.** — The permeability of stem tissues from the wilted region of infected shoots was determined 3 days

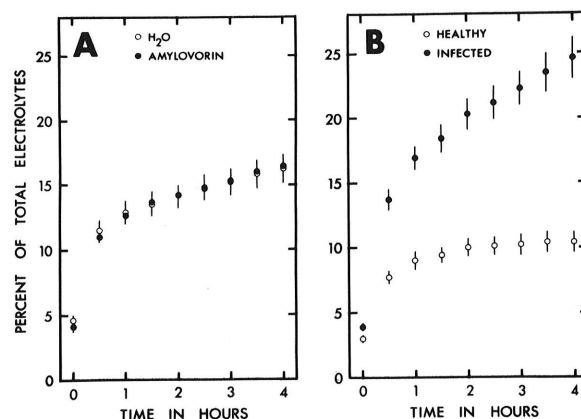


Fig. 4-(A, B). Electrolyte loss into 4 ml of water of four 5-mm *Cotoneaster pannosa* stem segments from A) nonwilted shoots in water or shoots wilted in 0.1 mg/ml amylovorin; and B) healthy control shoots or shoots infected with *Erwinia amylovora*. Data points are the mean of six replicates [five replicates of healthy control in (B)] and the vertical lines depict standard errors. Total electrolytes were determined by measuring the conductance of the bathing solution after freezing and thawing the samples.

after inoculation. Electrolyte loss from stem segments from infected shoots was much greater than that of segments from noninoculated shoots (Fig. 4-B). Infected tissues had lost more than twice as many electrolytes as had healthy tissues after 4 hr.

### DISCUSSION

The decreases in water potential, rate of water uptake and transpiration, and water conductance of stem segments observed in shoots exposed to amylovorin indicate that amylovorin induces wilting by restricting water uptake, rather than by disrupting membrane semipermeability or by inducing uncontrolled loss of water from leaves (26, 27). The similar appearance and water potentials of shoots wilted by amylovorin, dextrans, or blockage of shoot bases with wax further support the conclusion that amylovorin induces wilt by restricting water uptake.

The highly significant negative correlation observed between resistance to water movement in stem segments and the water potential of tissues distal to the segments (Fig. 3) suggests that resistance to water flow in stem xylem is an important factor in the wilting of cotoneaster shoots exposed to amylovorin. The mean resistance to water movement in stem segments from amylovorin-treated shoots was approximately three times greater than the mean resistance of segments from water-treated shoots. Increases in resistance of this magnitude have been considered too small to significantly affect tissue water potentials in most plants studied previously (7). However, the resistance to water movement observed in 10-cm segments of water-treated cotoneaster shoots (33 sec bar  $\mu\text{liter}^{-1}$ ) is much higher than the resistance observed in stem segments of similar length from other plants such as tomato (approximately  $3.0 \times 10^{-3}$  sec bar  $\mu\text{liter}^{-1}$ ) (5, 14). Thus, relatively small increases in xylem resistance of cotoneaster stems might be sufficient to markedly affect shoot water potentials. Furthermore, a restriction of water movement through stem segments suggests that similar restrictions may also occur in the xylem of the petioles and leaves (26). Therefore, increases in resistance to water movement in the total pathway from shoot base to leaf cell are likely to be higher than that indicated by measurements of only stem segment conductance.

Conductance of water in stem segments from amylovorin-wilted shoots did not increase as water was forced through stem segments. Thus, it would appear that the increased resistance observed in those segments results from an irreversible blockage of vascular elements rather than from transient viscosity effects of the polysaccharide solution. Although the site of blockage is unknown, direct occlusion of bordered pit membranes of xylem vessels has been suggested as a likely site of restriction of water movement by high-molecular-weight compounds (6).

Restriction of water movement in xylem by glycopeptides produced by *Ceratocystis ulmi* and *Corynebacterium insidiosum* has been postulated as the mechanism by which these pathogens induce wilting of their hosts (26, 27). Solutions of these high-molecular-weight compounds caused changes in shoot water re-

lations similar to those observed in shoots exposed to amylovorin. However, the water relations of shoots exposed to amylovorin were not comparable to those of shoots infected with *E. amylovora*.

The absence of wilting or decreased water potentials in apparently healthy tissues distal to tissues infected by *E. amylovora* indicates that significant restriction of water movement does not occur in the xylem of infected shoots. The increased electrolyte loss from infected stem tissues indicates that membrane integrity has been disrupted. Loss of membrane integrity of part or all of the succulent tissues enclosed in the pressure chamber would lower the balancing pressure required to force water in the xylem back to the cut surface of the shoot base (3), thus explaining the apparent higher water potentials of infected shoots. Localized disruption of membrane integrity in succulent stem tissues would result in their loss of turgor even though leaf tissue retained solutes normally and remained turgid.

The close association of bacteria to host cells showing the first indications of symptoms (1, 13, 15, 16, 18) suggests that factors involved in the early stages of pathogenesis are quite localized in their effect. Huang and Goodman (13) concluded that amylovorin was responsible for the initial ultrastructural changes observed in infected tissues, probably by affecting cellular membranes. However, the absence of increased electrolyte leakage from amylovorin-wilted tissues (Fig. 4-A) indicated that significant changes in membrane permeability had not occurred at the time wilting was apparent. We observed no directly detrimental effects of amylovorin on host cells that could explain the changes observed during the early stages of pathogenesis.

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