CHAPTER NINE

HYDRAULIC CONDUCTION AND WOUNDING RESPONSE OF *ACACIA AMOENA* STEMS KEPT IN CITRIC ACID AND IN DISTILLED WATER

9.1 INTRODUCTION

The importance of knowing the longest vessel length of the species in which hydraulic conduction is to be calculated cannot be overemphasised. The longest vessels are also the widest (Handley 1936), and therefore carry more water (Greenidge 1952). This relationship is shown in the Hagen-Poiseuille equation (Appendix L), in which flow volume is proportional to the fourth power of the capillary radius (Zimmermann and Brown 1971). In an intact plant, water must pass from one conducting element (collectively termed 'conduits', see Chapter 2, footnote) to another through pit membranes. Therefore, to imitate the *in vivo* situation, stem segments used in hydraulic conduction experiments should be longer than the longest vessel length. If stem segments are shorter than the longest conduit, the largest volume of water travels through the longest conduit unimpeded by pit membranes—akin to an open straw, or an electrical 'short' (Milburn 1979). In this situation, conduction rates are artificially exaggerated. Nevertheless, in several hydraulic conduction studies, very short stem segments have been used, e.g. roses, 2.5 cm (Gilman and Steponkus 1972); 4 cm (Durkin and Kuc 1966); 5 cm (Burdett 1970; Durkin 1979a; Put and van der Meyden 1988; van Doorn *et al.* 1989; van Doorn and Perik 1990) and *Thryptomene calycina*, 5 cm (Jones *et al.* 1993). Van Doorn *et al.* (1989) found that the maximum vessel length in 'Sonia' roses was 25 to 30 cm, but they only used 5 cm stem segments in hydraulic conduction experiments. Dixon and Peterson (1989) noted that at least 95% of conduits in *Rosa hybrida* 'Samantha' were less than 5 cm long, and so removed at least 5 cm from stems under water prior to experiments. However, such a procedure does not take into account the longest vessel length, which usually comprises only a small percentage of the total conduit number, but nevertheless may account for the greatest water flow (Milburn 1979). Although the maximum vessel length of *T. calycina*, a woody shrub, is not known, it is likely to be longer than 5 cm.

Indian ink has been employed for many years to determine conduit lengths (Handley 1936). Its use is based on the principle that the carbon 'ink' particles are larger than most pits (except *Abies* spp., Milburn 1979) which connect adjoining vessels, and thus will not be transmitted from vessel to vessel. Such a phenomenon enables the length of vessels that have been opened at the

cut end to be determined. Some later studies have used carbon-based paint (Skene and Balodis 1968) and also latex paint (Zimmermann and Jeje 1981; Middleton 1989) as an injection medium for determining conduit length. However, the disadvantage of using paint is that the larger surface tension effect of oil-based paints means that it is less likely to be pulled into small conduits at atmospheric pressure (J. Milburn, pers. comm. 1995).

The decrease in hydraulic conduction that occurs over time in cut flowers is a common observation that has been mentioned previously (section 4.1). Several studies have attributed the loss of hydraulic conductance to the deposition of plugging materials, such as callose and pectins ('gums'), in the xylem conduits (Pierson *et al.* 1955; Durkin and Kuc 1966; Burdett 1970; Parups and Molnar 1972; Lineberger and Steponkus 1976; Davies *et al.* 1981). Callose is a β-1,3 linked polysaccharide (Biggs 1985) found in sieve tubes and sieve plates of phloem tissue. It is frequently deposited after phloem wounding (Currier and Strugger 1956; Currier 1957; Englemann 1965) or pathogen invasion (Biggs 1984; de Leeuw 1985). Pectic substances are also polysaccharides (Baayen and Elgersma 1985), and form a major component of the middle lamella and primary cell walls (Lund 1982). In 1893, ruthenium red (ammoniated ruthenium oxychloride) was found to be specific for pectins (Mangin 1893), and since then has been widely accepted and used as a stain for pectic materials (Jensen 1962; Gurr 1965; Sterling 1970). Pectins, like callose, are also observed in xylem after pathogen infection (Powers 1954; Roth 1961; Sutton and Williams 1970; Baayen and Elgersma 1985) or wounding (i.e. cutting) (Fujino *et al.* 1983). Both callose and pectin deposition are thought to be general resistance responses which provide barriers to vascular infection (VanderMolen *et al.* 1977; Biggs 1984).

It is thought that pectic enzymes act simultaneously on the middle lamella and primary cell wall, solubilising and loosening them, respectively (Lund 1982). Some soft rot bacteria produce pectate lyases, which are enzymes involved in the degradation of cell walls (Dean and Wood 1967). However, pectate lyase secretion is not limited to the soft rot bacteria, as some non-phytopathogenic species degrade pectin without causing soft rot (Smith 1958). As mentioned previously (sections 4.4 and 7.1), *Pseudomonas* spp., commonly found in vase water, is one of the genera which synthesises pectate lyases. Burdett (1970) hypothesised that micro-organisms might secrete pectolytic enzymes which converted plant cell wall materials into vessel-plugging substances. Thus, it is possible that bacteria isolated from *Acacia* vase water could produce pectolytic enzymes, and cause blockage of xylem conduits.

The aims of this chapter were to:

- examine various injection media for the determination of conduit length, and then employ the best medium in order to obtain a reliable conduit length profile of *Acacia amoena*;

- determine the hydraulic conduction of excised *A. amoena* stems kept in distilled water and in citric acid;

- observe whether callose is formed in increasing amounts over time, and thus is involved in decreased hydraulic conduction in *A. amoena*;

- determine whether pectin is deposited in the xylem conduits of cut stems, and if so, whether this deposition increases over time; and

- test whether bacteria isolated from vase water containing *A. amoena* stems produce pectate lyase, and could thus be implicated in pectin deposition.

9.2 MATERIALS AND METHODS

9.2.1 Transmission electron microscopy of inks and paint

The particle sizes of several media were determined using transmission electron microscopy (TEM) in order to ascertain the best injection material for use in xylem conduit length measurement. Particle sizes were measured with a ruler from the resulting TEM photographs.

The particle sizes of two brands of indian ink: (1) Hunt's Speedball Super Black india ink (Hunt Manufacturing Co., U.S.A.); and (2) Rotring black indian ink (Rotring-Werke, Germany); and also the particle sizes of latex paint (Sunproof Gloss, Taubmans Pty. Ltd., Villawood, Australia; colour: Mid Bristol Green), were measured. A third ink, Pelikan Brilliant Black (Pelikan S.A., Spain) was unable to be measured as the particles could not be focused under the TEM in order to photograph them.

The ink samples were filtered three times (using a Buchner funnel) through Whatman No. 1 filter paper, and the filtrate was diluted to 1:40 with sterile distilled water. The paint was diluted to 1:100 (Zimmermann and Jeje 1981), then filtered three times through Whatman No. 1 filter paper; the filtrate was then centrifuged at 2,000 rpm for 30 min. A drop of each of the filtered, diluted inks and of the supernatant paint was placed on separate 200 mesh copper grids coated with 0.25% Formvar (Polaron, London) in chloroform. The excess ink was removed with filter paper and the drop was allowed to dry for a few minutes before being examined under a transmission electron microscope (JEOL JEM-1200EX, Japan Electro Optics Laboratory Co. Ltd., Tokyo, Japan) at 150,000× magnification and an accelerating voltage of 60 kV.

9.2.2 Determination of conduit length

Conduit length determination was based on Crombie *et al.* (1985b) and analysed according to Milburn and Covey-Crump (1971). Numerous initial attempts (in all, nine different attempts using various inks, paint and methods) were fraught with difficulties, evidenced by poor initial injection percentages of between 17 and 42%. Such low injection percentages lead to the TEM indian ink and paint studies described above (section 9.2.1). From those TEM studies, the best injection material appeared to be Hunt's indian ink (see Photographs 9.1 c, d), so it was used in all subsequent ink injection attempts. As a result of these injection problems, and after discussions with both J. Milburn and K. Ritman (pers. comm. 1991), it was concluded that many *Acacia* conduits had already cavitated *in situ*. Therefore, they needed to be injected forcibly to fill them with water, prior to indian ink infiltration.

Thus, conduit length determination using indian ink was preceded by vacuum infiltration of the stems. The experimental procedure was as follows: terminal shoots, 40 to 50 cm long were removed from the tree pre-dawn so as to ensure maximum water potential. Collection and hydration was as described previously (section 2.2). A large vacuum desiccator was then filled to just below the rim with distilled water. The stems were recut under distilled water, removing 2 cm from the base. The stems were wrapped in weighted chicken mesh. A lead ribbon was used to suspend the stems from a hook inside the lid of the desiccator, above the water level. Vacuum was applied (67 cm Hg = 0.12 bar below atmospheric pressure) for approximately 20 min to degass the water. The vacuum pump was then stopped, and the desiccator was rocked until the suspended stems fell into the water and were completely submerged (Milburn and McLaughlin 1974). The stems remained in the water for 24 h.

Vacuum infiltration being completed, 2 cm were removed from the base of the stems (under degassed water) to provide a fresh surface for ink injection. Hunt's indian ink was filtered three times through Whatman No. 1 filter paper and the filtrate was diluted 40 times with distilled water (Crombie *et al.* 1985b). The stems were placed in individual vials containing the diluted ink and Parafilm M was placed around the top of vials to prevent the ink from drying. A 60 W incandescent lamp was placed 30 cm from the stems, to promote transpirational pull as the stems drew up the ink.

Each stem was then sectioned by hand at 1 cm intervals. The sections were mounted in ethanol (70% v/v), and left for 5 min to allow any entrapped air bubbles within the xylem conduits to be expelled by the surface tension of the alcohol. This procedure ensured that air bubbles trapped within xylem conduits were not mistaken for ink-filled conduits. The number of ink-filled and clear xylem conduits was then counted at each 1 cm section.

9.2.3 Measurement of hydraulic conduction

Initial experiments to determine hydraulic conduction of cut vegetative *A. amoena* stems were performed using a potometer to measure the rate of solution uptake. However, this method proved to be too tedious and labour-intensive in view of the protracted length of time (5 d) over which experiments were performed. Therefore, hydraulic conduction was measured according to the method outlined in Williamson (1989). In this method, conduction is determined by the amount of solution taken up by a cut stem, as measured by weight loss on a four-place electronic balance (Model AC100, Mettler Instrumente Ag, Zurich, Switzerland). Terminal vegetative *A. amoena* stems, 50 cm long, were cut from the tree and trimmed to 35 cm under distilled water. The stems were enclosed in a plastic bag and kept in a beaker of distilled water overnight in a humid chamber to reach full turgor (section 2.2). While under water, leaves were removed from the stem, and the stem was recut at both ends to a length of 20 cm. The basal end of the stem was kept in water while the stem was dried with Kimwipes™ (Kimberly-Clark Australia Pty. Ltd., Sydney) and smeared with petroleum jelly (Snow White, Golden Fleece Petroleum, Australia). Defoliating and coating the stem with petroleum jelly ensured that transpiration rates were minimal. The stem was then fitted into the hydraulic conduction apparatus.

Rates of solution uptake were measured using the hydraulic conduction apparatus described in a flow chart (Fig. 9.1). The vacuum pump was set so that normal physiological pressure *in vivo*, i.e. 0.1 atm m^{-1} ($\cong$ 0.1 bar m^{-1} = 0.01 MPa) was replicated *in situ*. This was monitored by adjusting the pressure stabiliser (or "bleed") so that a column of Hg was raised to a height of 6.4 cm (i.e. a 3.2 cm change per arm) Plastic tubings led to the guard flask from the pressure stabiliser, the Hg manometer, and the stem. (Some of these tubings ensured against the entry of water into the Hg manometer, or a spill of Hg or water.)

The apical end of the 20 cm long stem segment was inserted into plastic tubing and Blu-Tack™ (Bostick, Emhart Australia Pty. Ltd., Sydney) was wound around the junction to ensure a complete seal. The basal end was suspended in a 50 mL beaker, which was constantly weighed by a four-place electronic balance. Parafilm "M" (American Can Company, Greenwich, Ct., USA) covered the beaker and the top glass door of the balance, except for a 5 mm^2 gap through which the stem passed. A retort stand and glass tubing to which the plastic tubing from the stem was attached ensured that no part of the stem touched the balance or beaker. An automatic timer (Fiber E59, Watson Victor, Sydney) and printer (Model GA 40, Mettler Instrumente Ag, Zurich, Switzerland) were connected to the balance. The timer was set to print out the weight registered on the balance at hourly (3600 s) intervals. The weight recorded every consecutive 12 h for 120 h was used. At the conclusion of each conduction experiment, evaporation losses from the beaker through the 5 mm^2 Parafilm gaps were determined for two consecutive 12 h periods. These readings were then averaged and subtracted from the 12 hourly weights. A light

source (100 W incandescent lamp (24 μmol m^{-2} s^{-1}) was placed 30 cm away from the stems to replicate the cavitation detection conditions (section 4.2.3).

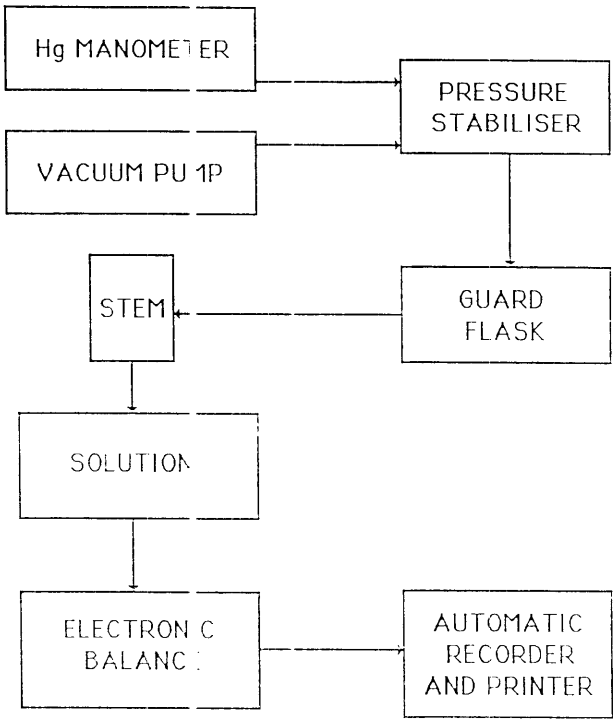


Fig. 9.1. Schematic representation of hydraulic conduction apparatus (from Williamson 1989).

9.2.4 Measurement of xylem conduit area

The *Acacia* stems used in hydraulic conduction experiments tapered slightly from base to top of the 20 cm long stem segments. Therefore, in order to obtain an accurate xylem conduit area, transverse sections were made by hand at the base, middle and top of the stems, using a new single-edged razor blade. Sections were mounted in glycerine (50% v/v) and examined under a light microscope connected to a computer, for image analysis. The area of each xylem conduit was measured using a computer image analysis program, Advanced BQ System IV (Bioquant System IV, R & M Biometrics Inc., U.S.A.). The xylem conducting area of each stem was calculated by obtaining the mean theoretical hydraulic conduction of the base, middle and top xylem conduit areas. Appendix K details the procedure for measurement of objects using the image analyser.

9.2.5 Calculation of hydraulic conduction

The equation used for the calculation of hydraulic conduction was that used by Dimond (1966), which he modified from Hagen and Poiseuille's (1839 and 1840 respectively, from Zimmermann and Brown 1971) equation describing laminar water flow through an ideal

(smooth) capillary. Dimond (1966) modified the Hagen-Poiseuille equation to account for flow through many capillaries of differing radii (Appendix L):

$$\text{Volume } (m^3\ s^{-1}) = \frac{\pi\Sigma(nr^4)p}{8l\eta} \qquad (9.1)$$

where
- r = radius of the capillary (m);
- p = pressure gradient along the capillary (Pa);
- η = viscosity of the liquid (kg m^{-1} s^{-1}); and
- l = length of capillary (i.e. stem segment) (m).

Hagen and Poiseuille found that liquid was stationary on the capillary walls (due to friction) and that its velocity increased towards the centre of the tube (parabolic flow). They noted that with parabolic flow, the flow rate was proportional to the fourth power of the capillary radius (Milburn 1979). However, it should be realised that the equations describe flow in *ideal* capillaries. Xylem conduits are far from being ideal capillaries because the inner walls contain, e.g. various types of secondary wall thickenings, pit membranes and, often, a warty layer (Liese 1965). These are all impediments to ideal flow and, as such, the Hagen-Poiseuille equation and its derivatives remain only a 'best estimate' of water flow at the present time. A detailed description of the method used to calculate hydraulic conduction is shown in Appendix L.

9.2.6 Callose and pectin deposition in excised *A. amoena* stems

The formation of callose and pectin in *A. amoena* stems was examined visually under a microscope. Hand sections were cut from separate stems every 12 h, stained and photographed over a 120 h period. Callose deposition was observed using decolourised aniline blue (0.005% w/v in 0.15 M K_2HPO_4, pH 8.2) and fluorescence microscopy (Currier and Strugger 1956; Currier 1957) between 334 and 365 nm λ with ultraviolet excitation, U exciter filter and a UV barrier filter Y475. [Currier (1957) reported that fluorescence microscopy was more sensitive than visible staining with light microscopy for the observation of callose.] Sections were stained for 10 min and then mounted in decolourised aniline blue (Currier 1957). Pectin deposition was observed under light microscopy after staining with aqueous (0.02% w/v) ruthenium red (BDH Chemicals Ltd., England) for 30 min, removing any excess stain in distilled water, and mounting the section in glycerine (50% v/v) (Gurr 1965).

9.2.7 Pectolytic activity of vase solution bacteria

The presence of pectolytic bacteria in the vase water after 5 d was determined using the method of Sands *et al.* (1972), except that antibiotics were not added to the medium because it was not

desired to select for fluorescent pectolytic pseudomonads. Streak plates were made, subcultured, incubated and assessed as described previously (section 7.2.7).

9.3 RESULTS

9.3.1 TEM analysis of inks and paint

Pelikan Brilliant Black ink, the particles of which were not photographed (and therefore not measured) did not appear to be a carbon-based (i.e. indian) ink because the particles were large and amorphous in comparison with the two other indian inks examined. The latex paint was also revealed to contain both large, amorphous and angular particles (Table 9.1; Photograph 9.1 a) with a mean size of 58 × 27 nm. These particles were larger than the particle sizes of the inks. Rotring indian ink contained spherical particles and had a mean particle size of 36.8 nm (Photograph 9.1 b). The best ink, in terms of particle uniformity, size and dispersal, was Hunt's india ink, which had a mean particle size of 33 nm (Photographs 9.1 c, d). Therefore, Hunt's india ink was used for ink injections to determine conduit length.

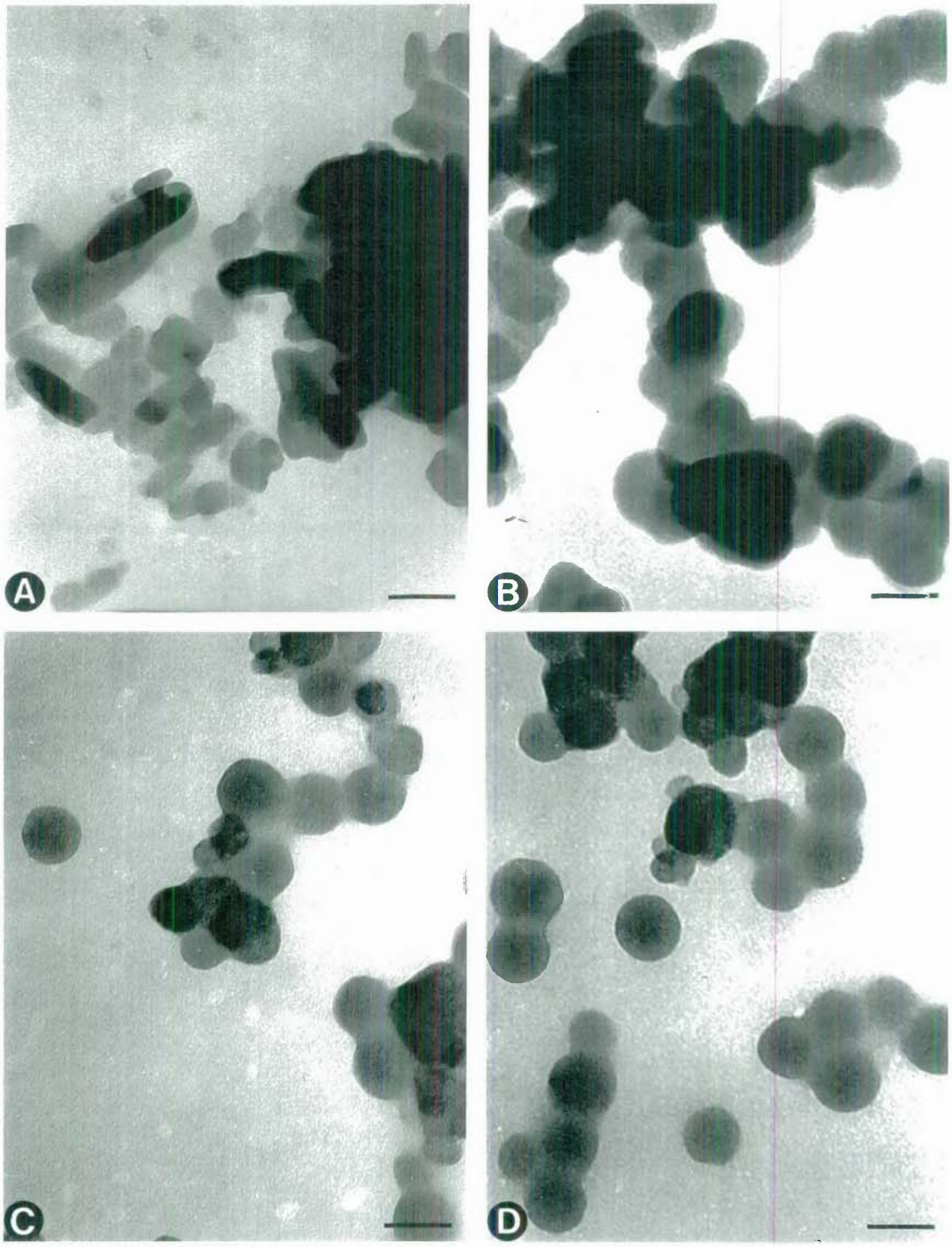
Table 9.1

Particle sizes of filtered, diluted latex paint and indian inks, as viewed under TEM

Medium	Particle shape	Mean particle size (nm)	Size range (nm)
Taubman's latex paint	rod-shaped and angular	58.16 × 27.04 (n = 20)	37.49 × 12.5 to 83.3 × 29.16
Rotring indian ink	spherica	36.80 (diameter) (n = 52)	24.99 to 49.98
Hunt's india ink	spherica	33.33 (diameter) (n = 119)	14.28 to 53.55
Pelikan ink	amorphous and rod-shaped (data not shown)	(not measured)	(not measured)

9.3.2 Conduit length profile of *A. amoena*

From the conduit length count of *A. amoena* stems (Fig. 9.2), it can be seen that the majority (50%) of xylem conduits are very short (0 to 1 cm). The longest conduit measured 10 cm, however, this accounted for only 0.2% of the total conduits. Even when vacuum infiltration was performed in order to maximise the number of conducting conduits, the mean injection rate at the basal end was 50%, indicating that 50% of conduits were permanently embolised or non-conducting in *A. amoena*.



Photograph 9.1. TEM photomicrographs of diluted latex paint and indian inks (bars = 50 nm). (**A**) Latex paint, diluted 1:100, ×120,000. Note rod-shaped and amorphous particles of varying sizes. (**B**) Rotring indian ink, diluted 1:40, ×150,000. The particles are spherical, but tend to agglomerate. (**C**) and (**D**) Hunt Speedball india ink, diluted 1:40, ×150,000. Note the well-dispersed, regularly shaped, small spherical particles.

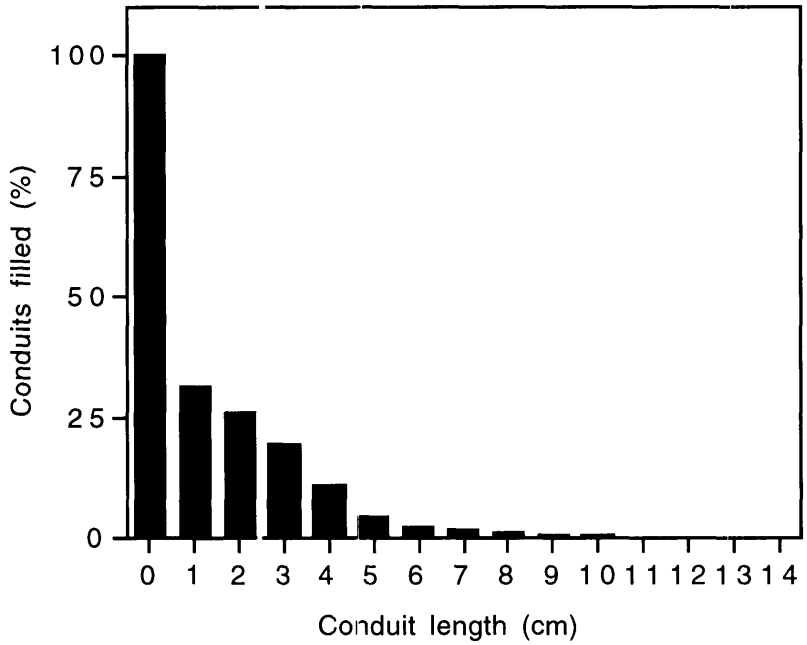


Fig. 9.2. Conduit length count of *A. amoena* stems. Stems were vacuum infiltrated to remove emboli and then injected with Hunt Speedball india ink. Transverse sections were cut by hand every 1 cm and the number of filled conduits was counted. The number of filled conduits per centimetre is expressed as a percentage of the number of initially filled conduits. Data are the means of five replicates.

When the conduit length distribution was analysed according to Milburn and Covey-Crump (1971), four classes of xylem conduits were evident (Fig. 9.3). The majority of conduits (74%) fell into the shortest (0 to 2 cm) class, 21.6% of conduits were in the 3 to 5 cm class, 3.6% were between 6 and 8 cm long, and 0.8% of conduits were between 9 and 11 cm long. (Appendix M details the total residual sum of squares of all possible linear regression allocations.)

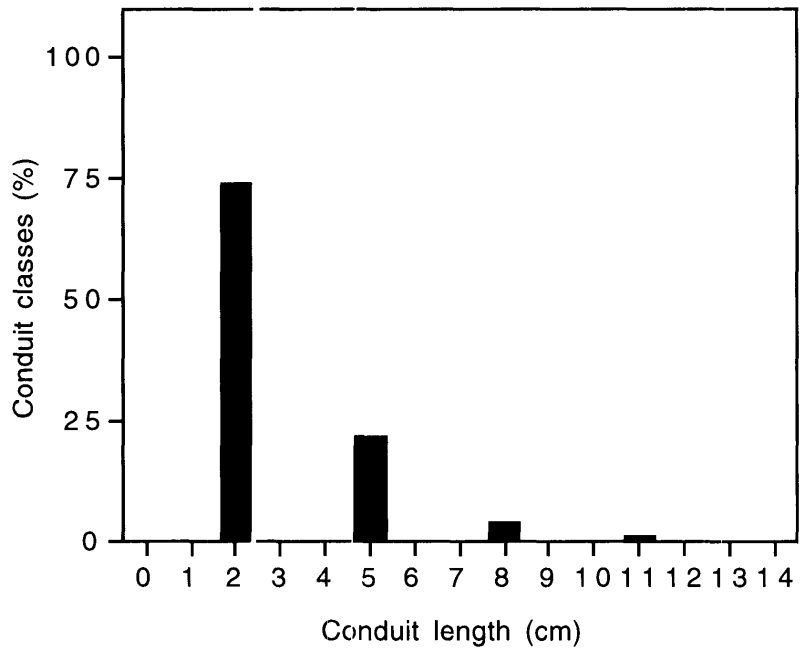


Fig. 9.3. Classes of xylem conduit lengths in *A. amoena* stems, as allocated by linear regression analysis according to Milburn and Covey-Crump (1971). (See Appendix M for the total residual sum of squares of all possible linear regression allocations.) Data are the means of five replicates.

An alternative method of conduit length allocation uses the direct conversion of counts to percentages (Zimmermann and Jeje 1981). For completeness, and for comparative purposes, the data described in Fig. 9.2 have also been analysed using this method (see Appendix N). Although the conduit length classes (Appendix N) were slightly different from those obtained using the Milburn and Covey-Crump (1971) method (Fig. 9.3 and Appendix M, A), the overall result was similar. In both methods, the largest percentage of conduits fell into the shortest conduit length class, and the smallest percentage of conduits belonged to the longest conduit length class.

9.3.3 Hydraulic conduction of *A. amoena* in distilled water and in citric acid (10 mol m^{-3})

The different results of xylem conduit areas from the base, middle and top of stem segments (Appendix O) emphasises the importance of obtaining an average of data from all these areas for hydraulic conduction analyses.

The rate of hydraulic conduction in distilled water was consistently lower than in citric acid (10 mol m^{-3}) throughout the experimental period (Fig. 9.4). However, there was only one significant difference ($P < 0.05$) between treatments within each 12 h period (Appendix C), which occurred at 36 h. After 24 h in distilled water, the rate of hydraulic conduction had fallen to 46% of the initial rate, whereas in citric acid it was 69% of the initial rate. After 120 h, hydraulic conduction rates were similar in both treatments, having fallen to 37% of the initial rate in distilled water and 39% in citric acid.

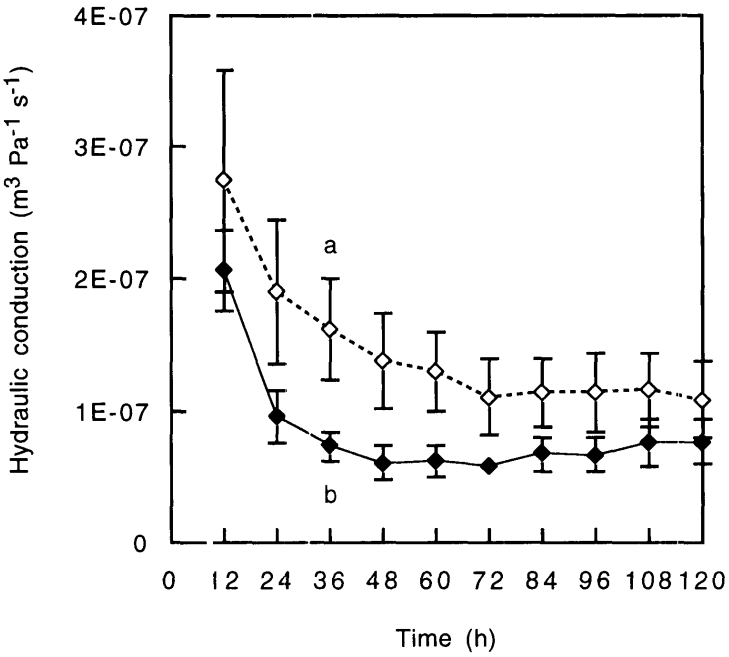
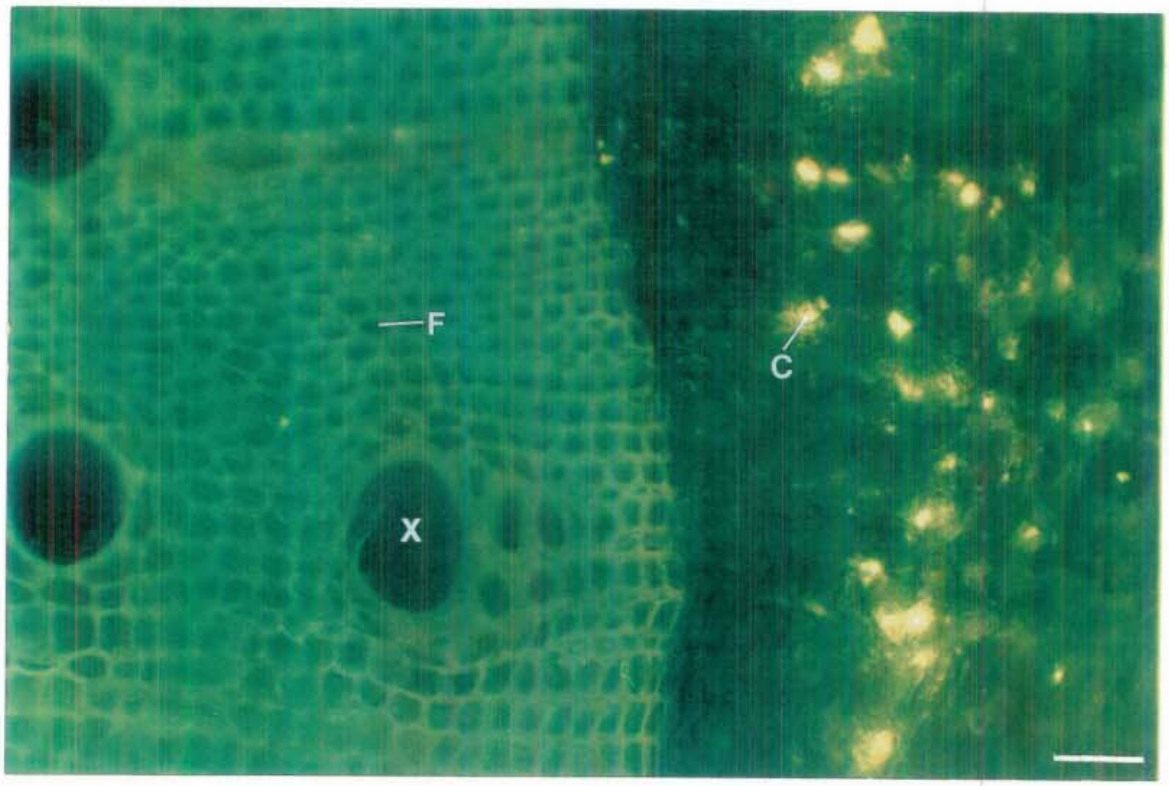


Fig. 9.4. The rate of hydraulic conduction in *A. amoena* stems kept in either distilled water (◆) or citric acid (10 mol m^{-3}) (◇). Within each 12 h period, there was only one significant difference between treatments ($P < 0.05$), which occurred at 36 h. (Where no error bar appears, the SE was smaller than the size of the symbol.) Data points are the means of five replicates per treatment.

9.3.4 Callose and pectin deposition in excised *A. amoena* stems

Callose deposition was limited to the phloem region (Photograph 9.2), and did not appear to increase or show any change during the 120 h experimental period (data not shown). The xylem region remained free of any fluorescent substance during the 120 h study period (apart from the slight autofluorescence of lignin in the conduit walls).



Photograph 9.2. Callose deposition in a transverse hand section cut immediately at the basal end of an *A. amoena* stem after 36 h in distilled water. Photograph taken using fluorescence microscopy. The light areas (e.g. C) indicate callose deposition. Note that callose is limited to the phloem region of the stem, and does not occlude any xylem conduits (X) or fibres (F) within the xylem region. There was no change in the deposition of callose during the experimental period, i.e. from 0 to 120 h (data not shown). (bar = 25 μm)

However, pectin deposition in the xylem conduits of stems kept in distilled water increased markedly over 120 h (Table 9.2; Photographs 9.3 to 9.5). No conduits were filled with pectin for the first 24 h; by 36 h 14% of conduits were filled, and by 120 h, 82% of conduits were filled with pectinaceous deposits. In contrast, no pectinaceous deposits were evident in the xylem conduits of stems kept in citric acid, even after 120 h (Photograph 9.6).

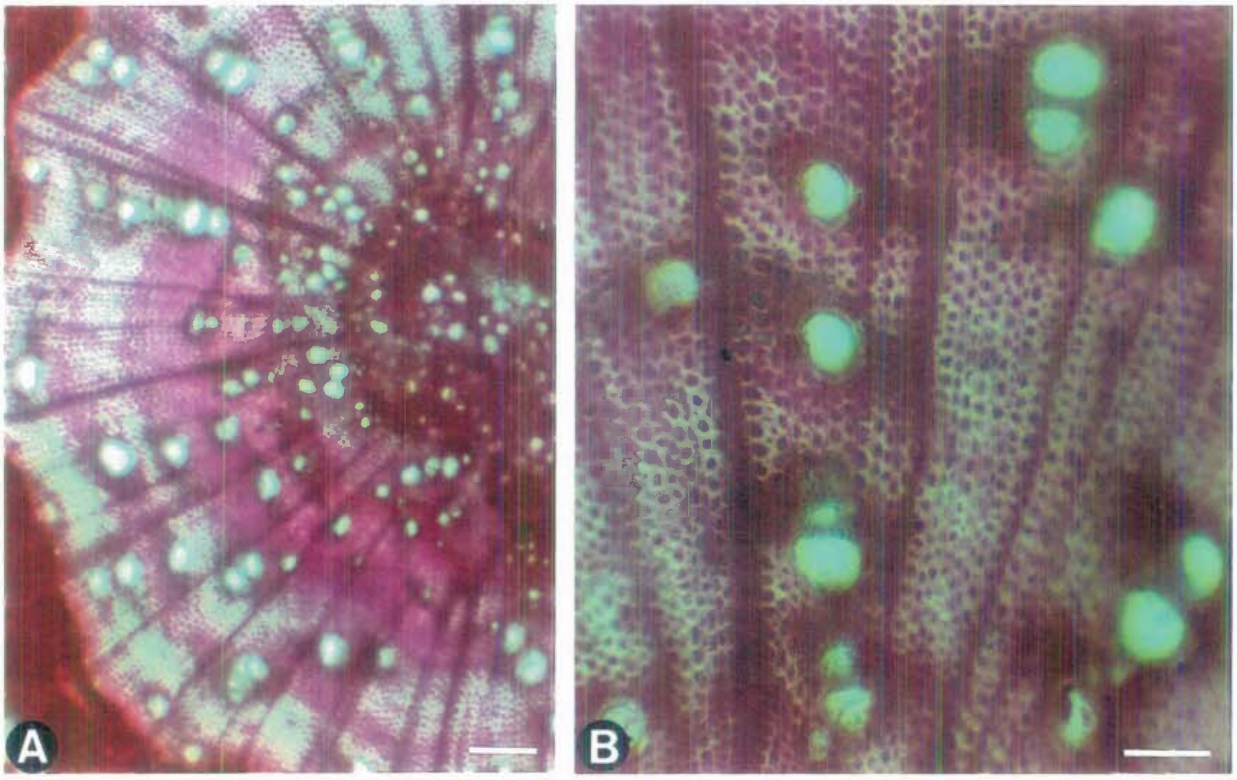
9.3.5 Pectolytic activity of vase solution bacteria

Bacteria from the vase water did not show pectolytic activity on the medium of Sands *et al.* (1972), as no clear zones were evident around the bacterial colonies (data not shown).

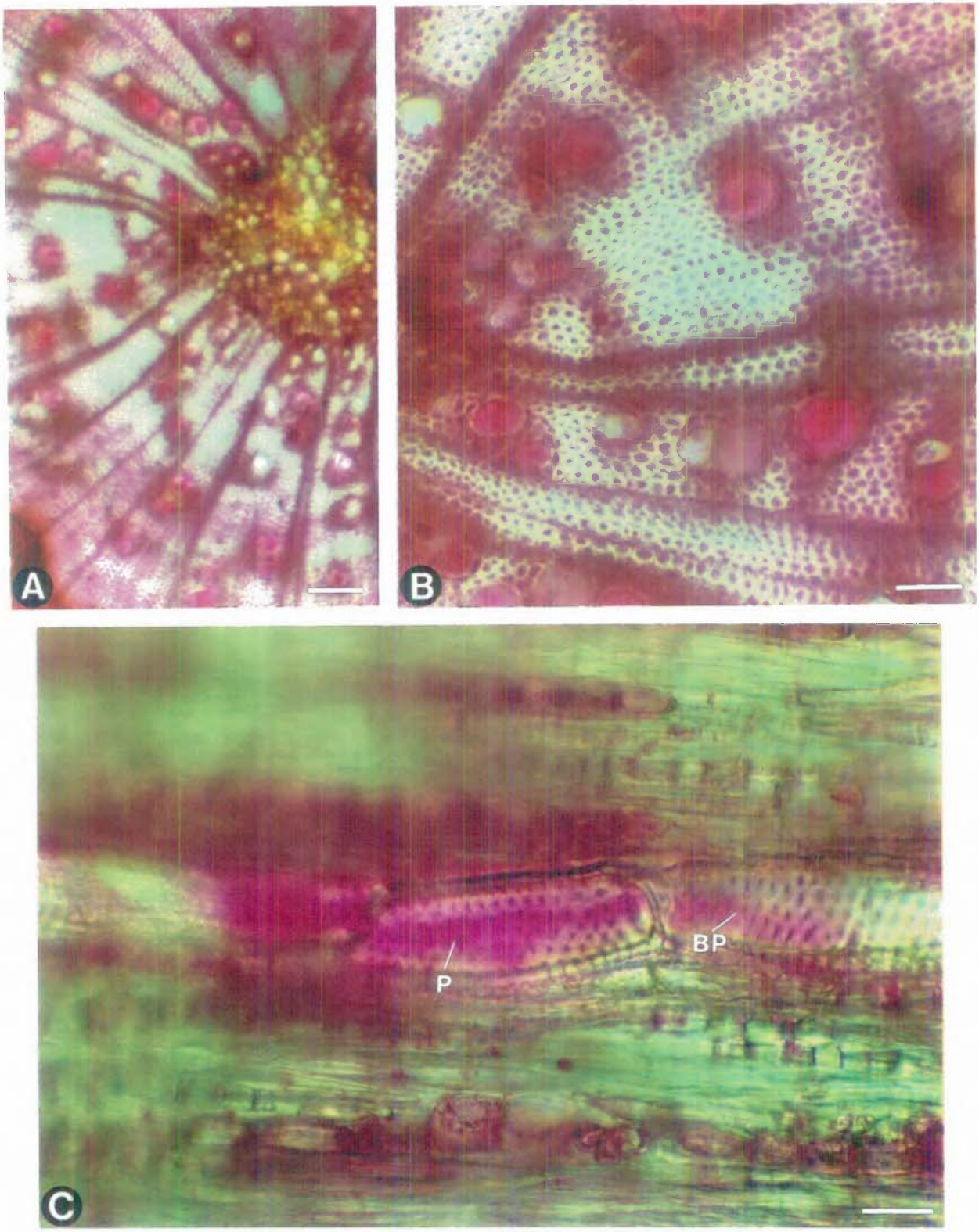
Table 9.2

The number of xylem conduits filled with pectinaceous deposits, as indicated by ruthenium red stain, in *Acacia amoena* stems kept in distilled water for 120 h

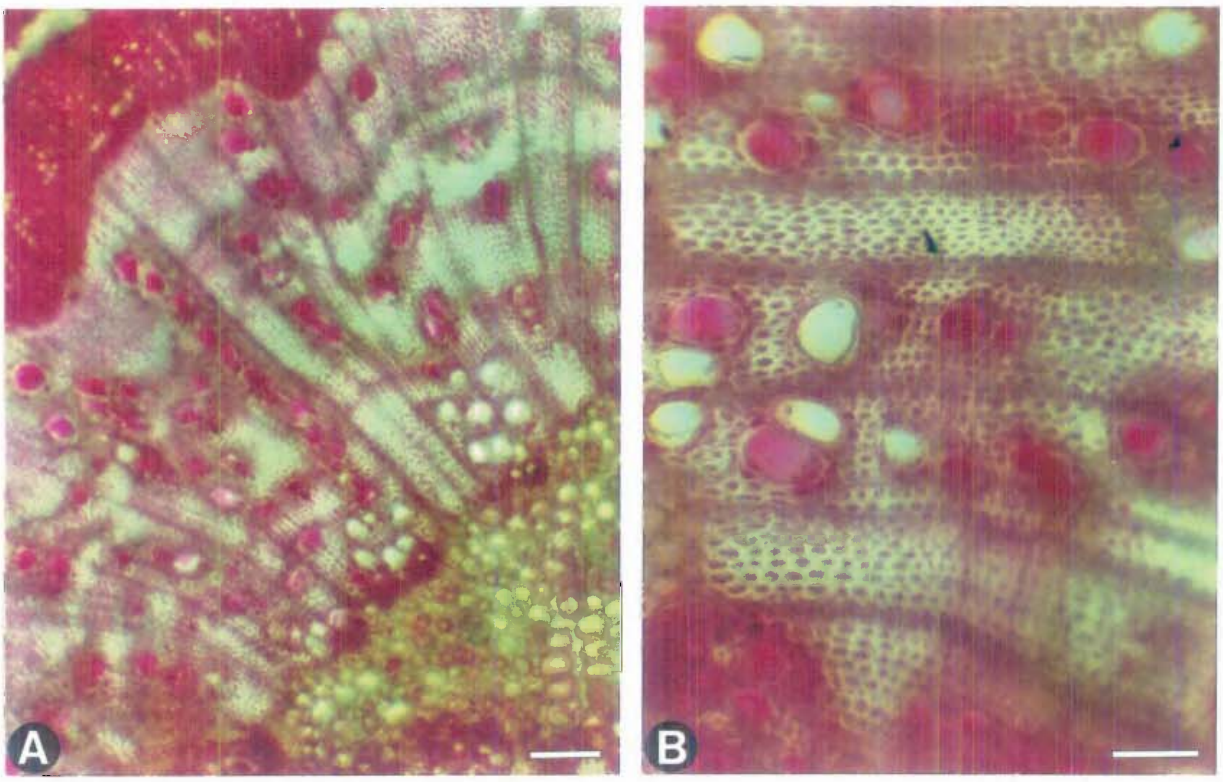
Time (h)	Conduits filled with pectin (%)
0	0
12	0
24	0
36	14
48	17
60	35
72	43
84	49
96	66
108	67
120	82



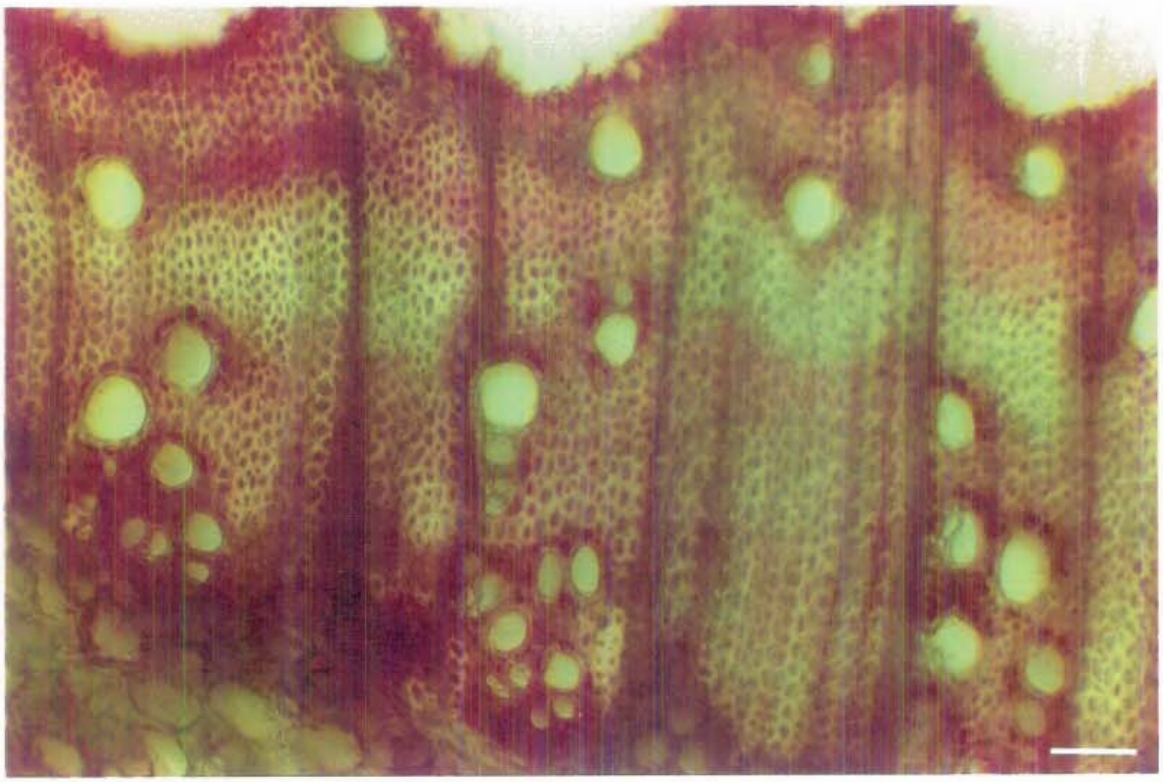
Photograph 9.3. A transverse hand section cut immediately at the basal end of a freshly cut (0 h) *A. amoena* stem stained with ruthenium red. Note that there is no deposition of pectinaceous materials in any of the xylem conduits. (**A**) Low power (× 40) (bar = 100 μm); and (**B**) high power (× 100) (bar = 50 μm).



Photograph 9.4. Hand sections cut immediately at the basal end of an *A. amoena* stem after 108 h in distilled water. Sections are stained with ruthenium red. (**A**) Low power (× 40) (bar = 100 μm) and (**B**) high power (× 100) (bar = 50 μm) transverse sections. Note that the majority of xylem conduit lumens are filled with a red stained substance, indicating deposition of pectinaceous materials. (**C**) Longitudinal hand section cut immediately at the basal end, showing red stained pectinaceous deposits (P) in a xylem conduit. Bordered pits (BP) are visible on the conduit wall. (× 200) (bar = 25 μm)



Photograph 9.5. A transverse hand section cut immediately at the basal end of an *A. amoena* stem after 120 h in distilled water. Section is stained with ruthenium red. Note that nearly all the xylem conduits are filled with red stained pectinaceous deposits. (**A**) Low power (× 40) (bar = 100 μm); and (**B**) high power (× 100) (bar = 50 μm).



Photograph 9.6. A transverse hand section cut immediately at the basal end of an *A. amoena* stem kept in citric acid (10 mol m^{-3}) for 120 h. Section is stained with ruthenium red. Note that all the xylem conduits are clear and do not contain any pectinaceous deposits. (bar = 50 μm)

9.4 DISCUSSION

Large differences in the various injection media were revealed by TEM (Table 9.1; Photograph 9.1). Ideally, the best injection medium is one in which the particles are well dispersed and of uniform size. Such properties reduce the risk of premature xylem conduit blockage (i.e. before the suspension reaches the end of the conduit). Hunt's india ink satisfied these requirements, and was used in all the conduit length studies reported in this thesis.

When the conduit length count (Fig. 9.2) was analysed according to Milburn and Covey-Crump (1971), the xylem conduit length distribution fell into four classes (Fig. 9.3). By far, the majority of conduits (74%) were very short (0 to 2 cm long), and only 0.8% of conduits were between 9 to 11 cm long. Such a finding is typical of conduit length distributions (Zimmermann and Jeje 1981; Middleton and Butterfield 1990), and emphasises the danger of employing the "95% method" of conduit length determination used by Dixon and Peterson (1989). The low percentage of long and wide conduits is thought to represent "safety versus efficiency" (Zimmermann and Milburn 1982), in which the long, wide conduits carry more water than narrower, shorter conduits, but are more prone to cavitation. However, it has been suggested that it is the size of pit membrane pores rather than the conduit diameter which determines a conduit's vulnerability to cavitation and subsequent embolisation (Tyree and Sperry 1989b). Hargrave *et al.* (1994) postulated that wider conduits would have larger and more frequent pores because of either: (1) a developmental link between conduit and pore diameter (Sperry and Tyree 1988); or (2) the sheer increase in number of pit membranes on wider conduits increasing the probability that some pits would have larger pores.

It should be pointed out that one of the assumptions of the Milburn and Covey-Crump (1971) method is that the conduit length distribution along a length of stem is constant, an assumption that may not hold in relation to (i) ring-porous wood, in which the early wood contains wider (and therefore longer) vessels than the late wood; or (ii) the part of the tree being sectioned (e.g. trunk compared with stem). Zimmermann (1978) found that there was a gradual decrease in conduit (vessel) diameter from base to top of the tree stem, and also from the stem to the small branches. Zimmermann and Potter (1982) observed that vessel length and diameter increased from twigs to branches to stem to roots. Thus, the validity of the method may well depend upon the time of year that the tree is sectioned (Handley 1936; Middleton and Butterfield 1990); whether the species is diffuse- or ring-porous; or the part of the tree being sectioned (Zimmermann and Potter 1982). Therefore, the present finding that 9 to 11 cm was the longest conduit class in *A. amoena* is only correct for terminal stems (up to 60 cm long) of the tree. Zimmermann and Jeje (1981) thought that the "complete randomness condition" would usually be met for shorter conduits (although the concept of "short" appeared to vary, depending on the longest conduit length in each genus). Thus, the Milburn and Covey-Crump (1971) method

may be suitable for conduit length analysis of *A. amoena*. It is is a diffuse-porous wood; and the longest conduit class was comparatively short (9 to 11 cm) compared with, e.g. ring-porous *Fraxinus americana* conduits, which are up to 18 m long (Greenidge 1952).

The present results for *A. amoena* conduit length compare favourably with those of Rappel (1985) and Ritman (1988) for *A. caesiella* Maiden et Blakely and *A. subulata* Bonpl., respectively. In *A. caesiella*, the maximum conduit length was in the 14 to 16 cm class, and in *A. subulata* the maximum length was between 12 to 13 cm. Such lengths are rather short compared with ring-porous trees such as *Quercus* and *Fraxinus* (Zimmermann and Jeje 1981), in which the longest conduits were approximately 10 m. However, acacias occur naturally in many drought-prone areas of Australia, in which such an internal safety system of shorter conduit lengths may have evolved as an hydraulic insurance against extremely negative water potentials and water shortages. Zimmermann and Milburn (1982) noted that plants subjected to greater water stress have smaller vessels than their more mesic counterparts because "smaller vessels are safer vessels". The external feature of adaptation to xeric habitats in many *Acacia* species is leaves which are reduced to glaucous phyllodes. This is the case in *A. amoena*, which also has other xerophytic characteristics such as a thick cuticle, a greater proportion of palisade to spongy mesophyll, a tightly-packed spongy mesophyll with few intercellular spaces (see Photograph 8.3), and a mid-rib vein surrounded by sclerenchyma fibres to provide mechanical strengthening against wilting.

Knowledge of the longest conduit in *A. amoena* was imperative for accurate hydraulic conduction results, as mentioned above (section 9.1). Stem segments 20 cm long were used to determine hydraulic conduction and, as the longest conduit was 10 cm (Fig. 9.2), the 20 cm stem length ensured that no conduit was open at both ends. This eliminated the possibility of obtaining spurious hydraulic conduction results, caused by water flowing through xylem conduits unimpeded by pit membranes. Nevertheless, it should be borne in mind that the rates of hydraulic conduction shown represent total <u>possible</u> conduction rates because in calculating the total xylem area, all xylem conduits were assumed to be conducting. The ink injection results (section 9.2.2) have shown that this is far from being the case in *A. amoena*.

The rate of hydraulic conduction in distilled water fell to less than half the initial rate after 24 h (Fig. 9.4). This coincided with a sharp decrease in xylem water potential and a rise in AAE production (Fig. 4.2). These results indicate that the few early AAE are important because they occur in conduits which carry a large proportion of water. Such a result has been found by other researchers (Tyree and Dixon 1986; Dixon *et al.* 1988; Ritman 1988). However, it should be noted that J. Milburn (pers comm. 1996) has observed (both acoustically and microscopically) that the very first AAE are produced by fibres, and not the larger, more vulnerable vessels, as is commonly thought. He believes that the fibres cavitate first to act as a

safety 'buffer' against cavitation in the more important vessels (and tracheids). Such an hypothesis would still accord with the above results because no obvious decreases in hydraulic conduction would be noticed after the initial fibre-produced AAE.

In citric acid, however, hydraulic conduction decreased at a slower rate than in distilled water (Fig. 9.4). A large decrease in xylem water potential occurred between 48 and 60 h in citric acid (Fig. 4.4), but this was not reflected in the hydraulic conduction rate between those times. Also, the number of AAE did not increase during the experimental period (120 h) (Fig. 4.4). The results of Chapter 5 revealed that citric acid may be degrading pit membranes, resulting in fewer available compartments in which cavitation could occur. Certainly, the loss of hydraulic conduction in citric acid was far more gradual than in distilled water, but treatment with citric acid seems to defy the usual quantitative measures of water stress. Nevertheless, the similar conduction rates at the end of the experimental period for stems in citric acid and in distilled water (Fig. 9.4) indicate that xylem blockage also occurs in citric acid, albeit at a slower rate than in distilled water.

Although the rate of hydraulic conduction was consistently higher in citric acid than in distilled water, there was only one significant difference ($P < 0.05$) between treatments. It is possible that more significant differences may have emerged if the number of replicates had been increased. However, the use of more replicates was precluded because the measurement of xylem conduit areas was a very time-consuming process, even with the aid of computerised image analysis.

The absence of any increase in callose production over time (section 9.3.5), and specifically, the lack of deposition within the xylem conducting tissue, indicates that the xylem blockage which occurs after cutting is not a callose-related wound response. Similar results were obtained for cut roses by Parups and Molnar (1972), who concluded that callose was not a component of xylem blocks.

In contrast with callose, pectinaceous deposits within the xylem conduits of stems kept in distilled water increased over time, as shown by the staining reaction with ruthenium red (Table 9.2; Photographs 9.3 to 9.5). Pectin deposition began after cut stems were in distilled water for 36 h, at which time 14% of conduits were pectin-filled. By 120 h, 82% of conduits were blocked by pectinaceous deposits. This appearance of pectinaceous blockages after 36 h corresponds with the increase in AAE production for stems in distilled water described in Chapter 4 (Fig. 4.2). In that experiment, AAE production increased markedly between 24 and 48 h. However, by the first sign of pectin deposition at 36 h, hydraulic conduction had fallen to 36% of the initial rate (Fig. 9.4). This indicates not only the importance of the few, early AAE events (Fig. 4.2), but also that pectin deposition, *per se*, does not cause decreased water flow.

Thus, it would appear that the beginning of pectin deposition occurs in response to xylem blockage, and is not the cause of the blockage. Perhaps pectin can only be deposited in xylem conduits that have recently ceased to conduct water, and in conjunction with a physiological or pathological blockage. Parups and Molnar (1972) noted that pectic substances appeared after physiological or pathological disturbance and were indicative of a blockage caused by cell wall breakdown. Yet, no pectic substances were deposited in stems that had been kept in citric acid, even after 120 h (Photograph 9.6). It is uncertain why this did not occur, because the rate of hydraulic conduction in citric acid was similar to that of distilled water after 120 h (Fig. 9.4). It is possible that the acidifying effect of citric acid affected pectin deposition through the inhibition of enzyme activity at low pH. Enzymes have a specific optimum pH at which their activity is maximal (Lehninger 1982).

Parups and Molnar (1972) found that pectic substances were a major component of xylem blockage in 5 d old rose stems, although, curiously, Dixon and Peterson (1989) did not. Pectic substances are lost from the middle lamella during cell wall hydrolysis (Webster 1968). 'Gels' which stained with ruthenium red, indicating pectins, were observed to originate from distension of the primary cell wall and middle lamella following vascular infection (VanderMolen *et al.* 1977). Fujino *et al.* (1983) observed vascular occlusions which tested positive for pectins in fern fronds kept in deionised water for 2 d. No occlusions were found in freshly cut fronds. This would indicate that pectin deposition is not an immediate wound response but, rather, a more gradual one, perhaps as a result of cell wall breakdown. However, under SEM, no change was observed in the appearance of rose xylem tissue after 3 d in various solutions (Rasmussen and Carpenter 1974). Even after 5 d in water, no changes were evident under SEM in *Acacia* or *Rosa* (Chapter 8). It is likely that the SEM is not sensitive enough to detect changes in cell wall and membrane porosity.

Durkin (1979a) thought that carbohydrate-type blockages were a consequence of loss of xylem function, and not the primary cause. He considered the loss of xylem function, from cavitation and subsequent embolisation, to be the cause of decreased solution uptake. However, the initiating factors in water column breakage were unknown (Durkin 1979a). Although cut roses kept in air for 1 d became severely wilted, no xylem conduit blockage was evident (Rasmussen and Carpenter 1974). However, embolised conduits would not be visible under SEM. Rasmussen and Carpenter (1974) thought that Burdett's (1970) work showed that blocking materials appeared after stem flow resistance increased. Their own work (1974) supports the hypothesis that blocking materials were not present until after water uptake decreased. They therefore hypothesised that vascular blockage by various materials was a secondary phenomenon exacerbated by reduced flow through the xylem (Rasmussen and Carpenter 1974). Gums and tyloses form when vessels become air filled, an observation made by von Reichenbach (1845 in Zimmermann 1979) and (unwittingly) confirmed by Chattaway (1949)

over 100 years later. Von Reichenbach (1845 in Zimmermann 1979) realised that tyloses and gums were the result, and not the cause, of the cessation of water conduction.

Gum deposits are frequently produced by members of the family Fabaceae, subfamily Mimosoideae, such as *Acacia* spp. and *Albizia julibrissin* (the silk tree), following injury or wounding (Phipps and Stipes 1976). *Acacia senegal* is used commercially as a source of gum arabic, a polysaccharide produced after injury to the tree. As mentioned in section 8.4, Chattaway (1949) noted that species with pit aperture diameters < 10 μm produced gums, and tyloses were formed in species with pit aperture diameters > 10 μm. However, tyloses were frequently observed in *Acacia* (Photographs 8.3 to 8.5), a species with pit aperture diameters < 10 μm (Williamson 1989; Photographs 8.1 c, d, e). In further contrast with Chattaway's (1949) hypothesis, tyloses and gums were both observed in the same xylem vessels of tobacco plants infected with *Phytophthora parasitica* var. *nicotianae* (Powers 1954).

Bacteria isolated from the vase water were not likely to be responsible for pectin deposition in xylem conduits because none of them synthesised pectate lyase. This finding is not uncommon, as relatively few bacteria are known to synthesise pectate lyases, although *Pseudomonas*, commonly found in vase water (Put 1990), is one of the genera which does (Nasuno and Starr 1966). However, such pectolytic pseudomonads are usually responsible for soft rot disease of vegetables, and pectolytic activity has not been detected in vase water bacteria. De Witte and van Doorn (1988) isolated nine strains (six species) of *Pseudomonas* from rose vase water and found that none degraded pectin. Therefore, they concluded that if bacteria were responsible for xylem blockage, it was unlikely to be through a bacterially-induced degradation of pectins in the cell wall. De Witte and van Doorn (1988) thus considered Burdett's (1970) hypothesis, that bacteria secrete [pectic] enzymes which digest cell walls, to be unlikely. Put and van der Meyden (1988) infiltrated cut roses with a non-pectolytic strain of *Pseudomonas putida* (10^6 cfu mL^{-1}) and found that mucoid or amorphic substances were deposited in and around xylem vessels and pits. This result lends further weight to the observation that vascular blockage is unlikely to be caused by enzymes secreted by bacteria.

The calcium ion, Ca^{2+}, is involved in the cross-linkage of pectic molecules in the cell wall (Ferguson and Drøbak 1988). One of the main constituents of the middle lamella is calcium pectate (Devlin and Witham 1983). Glenn and Poovaiah (unpublished, in Poovaiah 1988) noted that calcium protected the structure of the middle lamella from breakdown associated with senescence. Vase life has been extended by added calcium, both as a broadcast crop spray of $CaCl_2$ to chrysanthemums (Siti Aishah and Yenni Erliana 1992), and in roses as a vase solution additive, either as $Ca(NO_3)_2$ or $CaCl_2$ (Michalczuk *et al.* 1989). Ca^{2+} has been assigned many roles, not the least being its importance as a secondary messenger (Hepler and Wayne 1985). However, its role in senescence processes is perhaps related to its ability to maintain both

membrane and cell wall integrity (Poovaiah and Leopold 1973). Thus, it is possible that additional calcium extends vase life by decreasing cell wall breakdown, so perhaps less pectin blockage occurs in the xylem of cut flower stems treated with calcium. However, this hypothesis remains to be tested.

Finally, a wound reaction, suberisation, not considered previously in the impairment of cut flower water uptake, must be discussed for completeness. Suberin is a fatty substance that is impermeable to water. It is found naturally lining the radial and transverse walls of the endodermis, and in cork cells of the phellem layer. However, it is also formed after injury and in response to pathogen invasion (Appel 1906 in Smith and Smart 1955; Zucker and Hankin 1970b). It is thought to assist in the effectiveness of compartmentalisation after wounding (Appel 1906 in Smith and Smart 1955; Smith and Smart 1955; Cline and Neely 1983; Schmitt and Liese 1993). Wounded potato tissue formed a suberised layer within 24 h of injury, which protected the tissue from attack by pectic enzymes produced by bacteria (Zucker and Hankin 1970a). Suberin deposition was also the first wound response observed within 24 h in geranium cuttings (Cline and Neely 1983). Cycloheximide, an inhibitor of protein synthesis, reduced suberin formation and hence decreased resistance to pathogen invasion (Zucker and Hankin 1970a). Cycloheximide has also been found to increase the longevity of cut carnations (Dilley and Carpenter 1975; Drory *et al.* 1995), daylily (Lukaszewski and Reid 1989; Lay-Yee *et al.* 1992), *Gladiolus*, *Iris* and *Narcissus* (Jones *et al.* 1994), although the probable decrease in suberin formation was not discussed. Rather, these authors ascribed the beneficial effect of cycloheximide to inhibition of protein synthesis. Deposition of a suberised layer in response to the wounding occasioned by harvesting cut flowers is an attractive hypothesis to explain decreased solution uptake over time. However, the beneficial effect of cycloheximide appears to be only temporary. Milburn and Williamson (1996) found that cut privet (*Ligustrum vulgare* L.) stems infused with cycloheximide (100 mol m^{-3}) had a higher rate of hydraulic conductance than controls, but conductance declined in both treatments over time. Perhaps if the experiment were conducted for more than 20 h, a suberised layer could develop fully in the control stems and a significant increase in conduction would be apparent for a longer time in cycloheximide-treated stems. Nevertheless, because of its toxicity to humans, cycloheximide is only beneficial as a research tool to determine whether suberin synthesis is responsible for the initial cause of decreased hydraulic conductance.