CHAPTER EIGHT

ANATOMICAL AND MICROBIAL CHANGES DURING VASE LIFE: SCANNING ELECTRON MICROSCOPIC OBSERVATIONS OF *ACACIA AMOENA* AND *ROSA HYBRIDA* 'SONIA'

8.1 INTRODUCTION

Scanning electron microscopy (SEM) provides excellent three-dimensional images of surfaces and, as such, is an ideal tool with which to study the details of biological specimens such as plants. The column and specimen chamber of the scanning electron microscope are under considerable vacuum (10^{-5} Torr). Therefore, biological specimens need to be dehydrated prior to SEM observation to prevent the vapourisation of hydrated tissue from causing specimen distortion, movement, and hence, mage degradation (Wilson and Robards 1984). Several methods of preparing material for SEM observation exist, the most common of which are: air drying (AD, e.g. using OsO_4 vapour); freeze drying (FD, the drying of a frozen specimen via the sublimation of ice under vacuum at low temperature); and critical point drying (CPD, material is usually chemically fixed, dehydrated, then dried in a critical point drying chamber in which the vapour density becomes the same as the liquid phase density, thereby eliminating surface tension effects).

However, each of these SEM preparation methods has inherent artefactual problems. Nevertheless, CPD and FD are the most commonly used preparation methods (Lamb and Ingram 1979), although CPD is considered the superior method (Meller *et al.* 1973). AD is thought to produce unacceptable results because the high surface tension forces as water evaporates into air can cause shrinkage, structural collapse and distortion (Anderson 1951; 1953a, b; Lamb and Ingram 1979). However, Read *et al.* (1983) thought that the disadvantages of slow (air) fixation may be offset by preservation of labile components on the specimen surface, which may otherwise be removed during solution immersion. AD with OsO_4 vapour produced excellent images of aerial fungal structures compared with, *inter alia*, FD and CPD (Quattlebaum and Carner 1980). During FD, two phase boundaries move through the specimen: (i) the liquid-solid boundary during freezing; and (ii) the solid-gas boundary during sublimation (Anderson 1951; 1953a). Both boundaries may cause specimen damage (Parsons *et al.* 1974). Whilst FD reduces tissue shrinkage, ice crystal formation can produce artefacts (Boyde 1978; Lamb and Ingram 1979) and distortions (Falk *et al.* 1971). In CPD, the capacity of liquid in the specimen to form fluid phases is eliminated, thus removing surface tension effects (Anderson

1951). However, the ethanol dehydration process which frequently precedes CPD has been found to remove fungal vegetative hyphae and sporulating structures (Quattlebaum and Carner 1980). Tissue shrinkage can also occur during dehydration prior to CPD (Lamb and Ingram 1979). The largest amount of tissue shrinkage is believed to occur during AD, followed by CPD, with FD producing the least shrinkage (Boyde 1978; Read *et al.* 1983).

Read *et al.* (1983) compared several SEM preparation techniques, including those mentioned above, and concluded that "no method of preparation surpasses all others". They recommended that several preparation methods be employed for comparative purposes, and that the examination of specimens under low temperature (cryo-SEM, or frozen-hydrated, FH) be considered as a benchmark for comparative purposes. The frozen-hydrated (cryo-SEM) method, as the name suggests, enables the examination of frozen specimens to be made without the need for prior dehydration. Thus, many artefacts, such as shrinkage and mechanical surface damage caused by the dehydration process and chemical fixation, are eliminated. Many researchers consider the cryo-SEM method to be superior to other preparation methods (Nei *et al.* 1971; 1973; Booij *et al.* 1992), particularly for the observation of microbiological material (Beckett and Read 1981; Read *et al.* 1983; Fraser and Gilmour 1986). Cryo-SEM images produced more defined and detailed microbial structures than conventional SEM preparation methods (Put *et al.* 1991; van Doorn *et al.* 1991b).

The search for the cause of xylem blockage in cut flowers has lead to a considerable number of SEM studies, aimed at detecting anatomical and microbiological changes during vase life (Rasmussen and Carpenter 1974; Put and Clerkx 1988; Put and van der Meyden 1988; Clerkx *et al.* 1989; Put and Rombouts 1989; Put *et al.* 1991; van Doorn *et al.* 1991a, b; van Doorn, de Stigter, de Witte and Boekestein 1991; Put *et al.* 1992). No such studies have been conducted on the genus *Acacia*. However, they could provide insight into the causes of ephemerality of this cut flower. SEM examinations of *Rosa hybrida* 'Sonia' are frequent (Put and Clerkx 1988; Put and van der Meyden 1988; Clerkx *et al.* 1989; Put and Rombouts 1989; Put and Klop 1990; Put *et al.* 1991; van Doorn *et al.* 1991b; van Doorn, de Stigter, de Witte and Boekestein 1991; Put *et al.* 1992), and provide useful comparisons with the SEM preparation methods used in this study of *Acacia*. However, many of the *Rosa* SEM studies have revealed the presence of "amorphous" deposits in the xylem (Put and Clerkx 1988; Put and van der Meyden 1988; Put and Rombouts 1989; van Doorn *et al.* 1991 b; van Doorn, de Stigter, de Witte and Boekestein 1991). The identity and function of these deposits is uncertain. It is not known whether *Acacia* would contain similar deposits.

Thus, the aims of the experiments reported in this chapter were to:

- obtain an understanding of *Acacia amoena* vascular anatomy;

- determine if any anatomical or microbiological changes would become evident at the SEM level during the vase li'e of *A. amoena* and *Rosa hybrida* L. 'Sonia';

- compare four different SEM preparation methods of *R. hybrida* 'Sonia' stems for image quality and ease of bacterial c bservation; and

- elucidate the puzzling incid:nce of varied deposits, collectively termed "amorphous", frequently observed in cut st:ms viewed under SEM.

8.2 MATERIALS AND METHODS

8.2.1 Plant material

Acacia amoena plant material w:.s obtained from the Botany Department garden, UNE, Armidale, as described previously (section 2.1). *Rosa hybrida* 'Sonia' flowering stems were obtained from a local florist, as d:scribed earlier (section 7.2.1). Plants were kept under standard vase life conditions (sectio:.s 2.7 ar.d 2.8). There were five replicates per treatment.

A. amoena stems were kept in two tieatment solutions: (i) distilled water; and (ii) an STS pulse (0.5 mol m^{-3}) for 16 h, then into c tric acid (10 mol m^{-3}) and SDIC (50 mg L^{-1}). The latter solution was found previously to prc duce a longer vase life than all other treatments (Table 3.3). Also, the silver ion and chlorine ha'e well known antimicrobial properties, so would provide a contrast with stems kept in distilled 'vater.

8.2.2 Maceration

In order to determine the nature of *A. amoena* fibres, plant material was macerated using a technique based on the method of S trelis and Green (1962). Stems were cut into 10 mm long segments and then into 0.5 mm wic ths with a sharp scalpel, placed in capped vials containing Jeffrey's solution (10% nitric and 1)% chromic acids) and heated to 40°C on a hot plate. The vials were shaken periodically to determine whether the woody elements had separated. Maceration was hastened by changir g the solution every 2 d, and was complete after 4 d.

8.2.3 Scanning Electron Microscopy

Air dried (AD) material

On days one, three and five of vase life, 5 mm transverse sections were removed from the basal end of different *R. hybrida* 'Sonia' stems using a new single-edged razor blade. These segments were then cut in half longi udinally and air dried with osmium tetroxide (OsO_4) vapour (2%), following the method of Reac *et al.* (1983). The specimens were mounted on aluminium

stubs with Supa Glue® (Selleys, Australia) and stored in a desiccator before being sputter coated for 4 min with 20 nm gold.

Critical point dried (CPD) material

Stem segments of *R. hybrida* 'Sonia' and *A. amoena* were harvested and cut as above, then prepared for SEM following modified methods of Allen (1981) and Woodland (1982). Briefly, the methods were as follows (modifications are shown in bold type). Specimens were fixed in 3% glutaraldehyde in 0.1 M Sörenson's phosphate buffer for **24 h** at 4°C; post-fixed in 2% OsO_4 in 0.1 M Sörenson's phosphate buffer for **15 h**; and dehydrated in 50, 70, 80, 90 and **95**% ethanol for 10 min for each concentration, followed by two changes in 100% dried ethanol of 15 min each. (No dehydration step of 30% ethanol was used. A detailed description of the preparation method is given in Appendix I.)

Freeze dried (FD) material

Stem segments of *R. hybrida* 'Sonia were harvested and prepared as above. They were placed in 1.5 mL Eppendorf (Germany) micro centrifuge test tubes containing 0.5 mL of 4°C sterile distilled water. The tubes were placed in a brass holder in a freezer for 24 h before being transferred to the arms of a freeze drier for 72 h. Specimens were mounted on aluminium stubs and stored in a desiccator until they were sputter coated with gold as described above.

Specimens prepared using the above methods were examined under a scanning electron microscope (Model JEOL JSM35, Japan Electro Optics Laboratory Co. Ltd., Tokyo, Japan) at an accelerating voltage of 15 kV.

Cryo-Scanning Electron Microscopy

This technique was used only as a trial with a two day-old stem of *R. hybrida* 'Sonia'. The method used was outlined in the JEOL JSM35 manual (Appendix J) and employed a Cooling Stage (35-CS2), maintained at -130°C with liquid air. Specimen observation was at an accelerating voltage of 8 kV. The specimen could not be sputter cryo coated or etched, which are conventional cryo-SEM procedures, because neither cryo transfer nor etching equipment were available at the University.

8.2.4 Light microscopy

Plant material was hand sectioned using a new single-edged razor blade, and mounted in 50% glycerine for examination under a light microscope (section 2.3).

8.3 RESULTS

8.3.1 Anatomy of *A. amoena*

Stem surface

The cuticular surface of young, terminal shoots appears undulated when seen under light microscopy (Photograph 4.3) and, especially when viewed under SEM (Photograph 8.1 a). The surface depressions provide ideal shelter for phylloplane fungal hyphae (Photograph 8.1 a). Lange (1969) described these undulating cuticular formations (seen in *Acacia melanoxylon*) as "convexities".

Stomates

A. amoena stomates are of the paracytic (formerly known as rubiaceous) type (Metcalfe and Chalk 1950; Wilkinson 1979), as can be seen in Photographs 8.1 b and 8.2 of the abaxial phyllode surface. Stomates of this type are characterised by having one or more subsidiary cells which run parallel to the long axis of the pore and guard cells. The stomate in Photograph 8.1 b is open as the two large kidney-shaped guard cells have pulled away from each other to expose the stomatal pore below. Although the classification of *Acacia* stomatal types as paracytic (rubiaceous) is not as straightforward as previously thought (Metcalfe and Chalk 1950), paracytic stomata are the most common type found in phyllodinous acacias (Grosso *et al.* 1994).

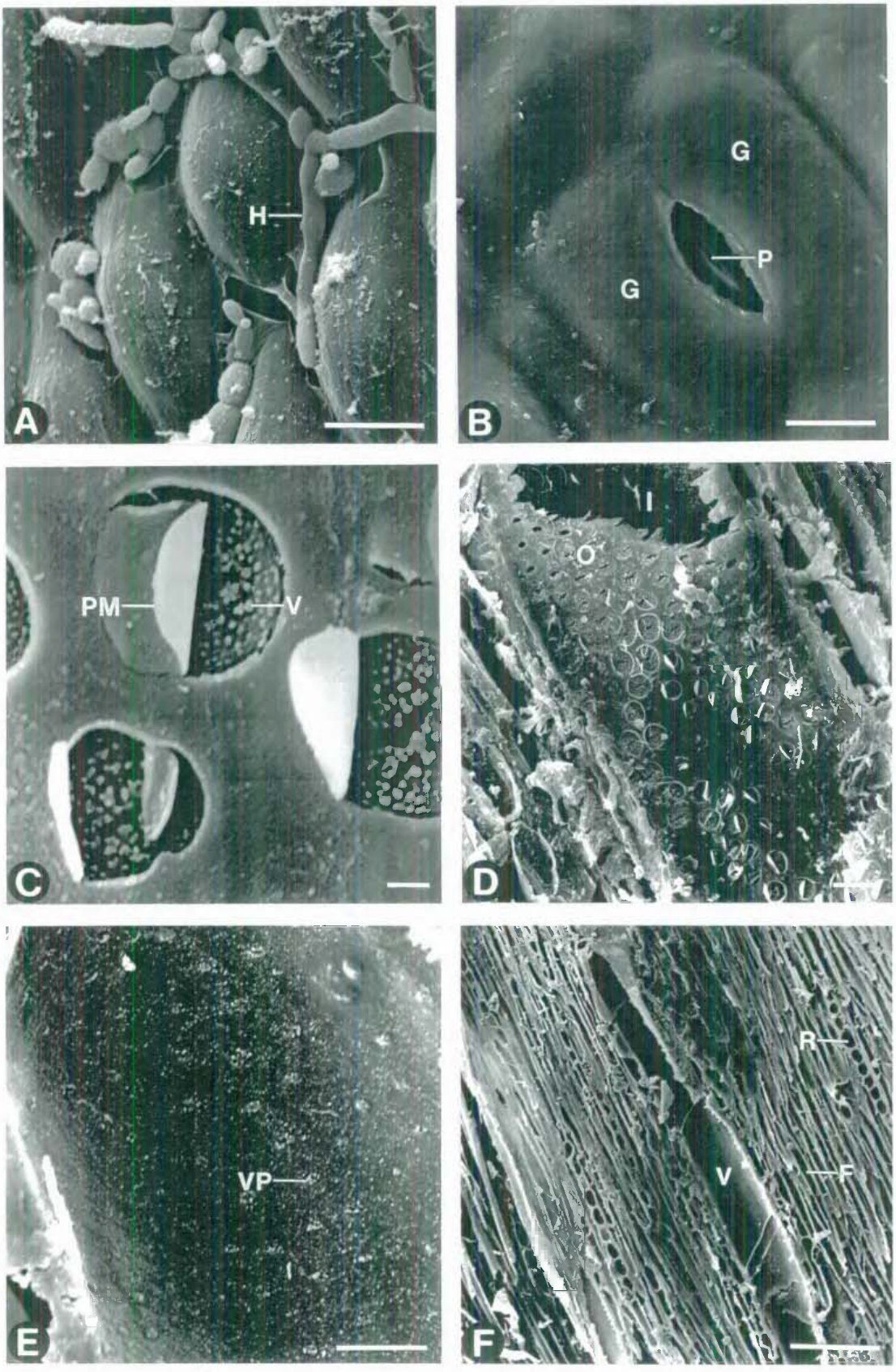
Leaf anatomy

Transverse sections of *A. amoena* phyllodes (Photograph 8.3) help reveal the xeromorphic characteristics of this plant. These include glaucous isobilateral leaves which have been reduced to phyllodes (Photograph 4.1), stomata protected by an outer stomatal ledge, a thick cuticle, a higher percentage of palisade (approx. 49%) to spongy (approx. 34%) mesophyll, spongy mesophyll with few air spaces (Photograph 8.3), and abundant sclerenchyma which form girders around the vascular bundles (data not shown) to provide structural support against wilting (Metcalfe and Chalk 1983).

Pits

A. amoena conduits have bordered vestured pits (Photographs 8.1 c, d, e). Photograph 8.1 c shows three bordered vestured pits on the outer wall of a xylem vessel. The location of these three vestured pits is shown in Photograph 8.1 d. During specimen cutting, the razor blade fortuitously cut between two adjacent xylem vessels, stripping the pit membrane and so exposing the vestures below, on the outer vessel wall (Photographs 8.1 c, d). Vestured pits as they appear on an inner vessel wall are shown in Photograph 8.1 e. The location of this vessel is shown in Photograph 8.1 f. The vessel is surrounded by numerous fibres and some ray cells.

Photograph 8.1. SEM photomicrographs (prepared by CPD) depicting some anatomical features of *A. amoena*. (**A**) Surface of a terminal shoot. Note the undulating nature of the young stem, the depressions of which provide shelter for phylloplane fungal hyphae (H) (bar = 10 µm). (**B**) The abaxial phyllode surface, showing a stomate of the paracytic type. The stomate is open as the two large, kidney-shaped guard cells (G) have pulled away from each other to reveal the stomatal pore (P) below (bar = 10 µm). (**C**) Three bordered, vestured (V) pits on the outer wall of a xylem vessel. The pit membrane (PM) is peeled back, exposing the vestures below (bar = 1 µm). (**D**) As (**C**), but lower magnification to show location. (O) = outer vessel wall, (I) = inner vessel wall (bar = 10 µm). (**E**) The appearance of vestured pits (VP) on an inner vessel wall (bar = 10 µm). (**F**) As (**E**), but lower magnification to show location. The vessel (V) is surrounded by numerous fibres (F) and some ray cells (R), tangential section (bar = 100 µm).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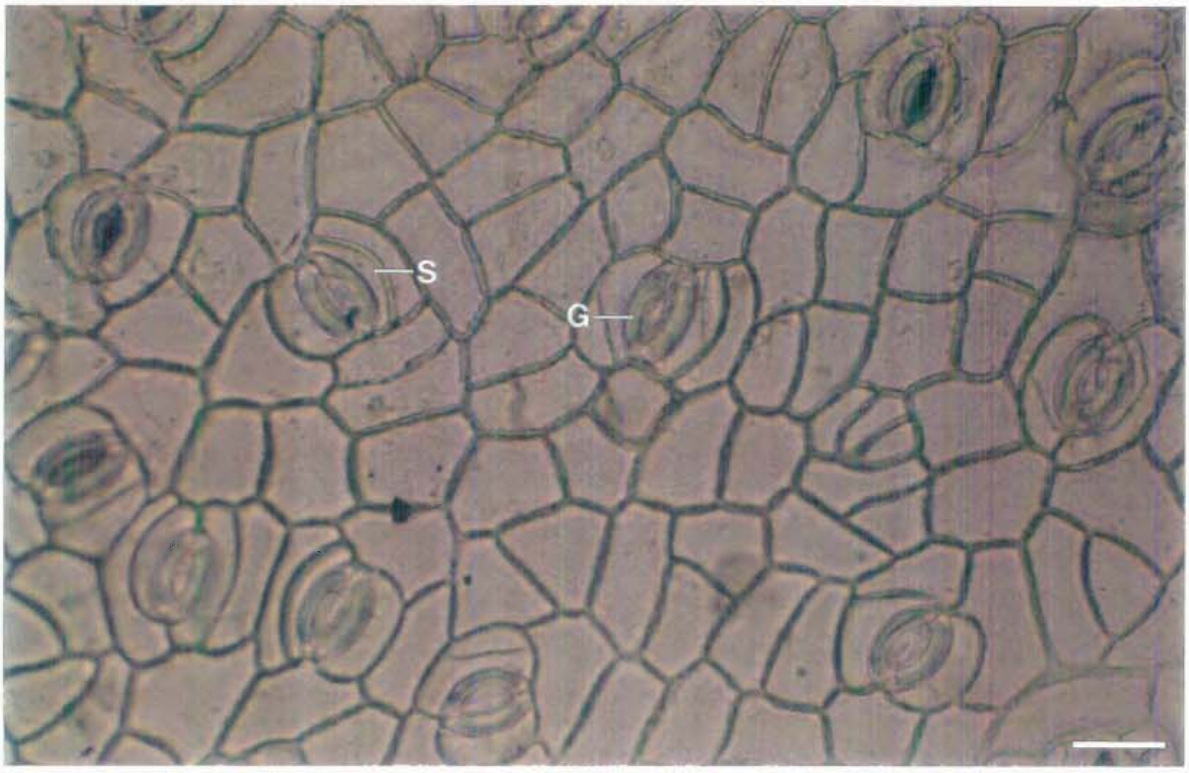
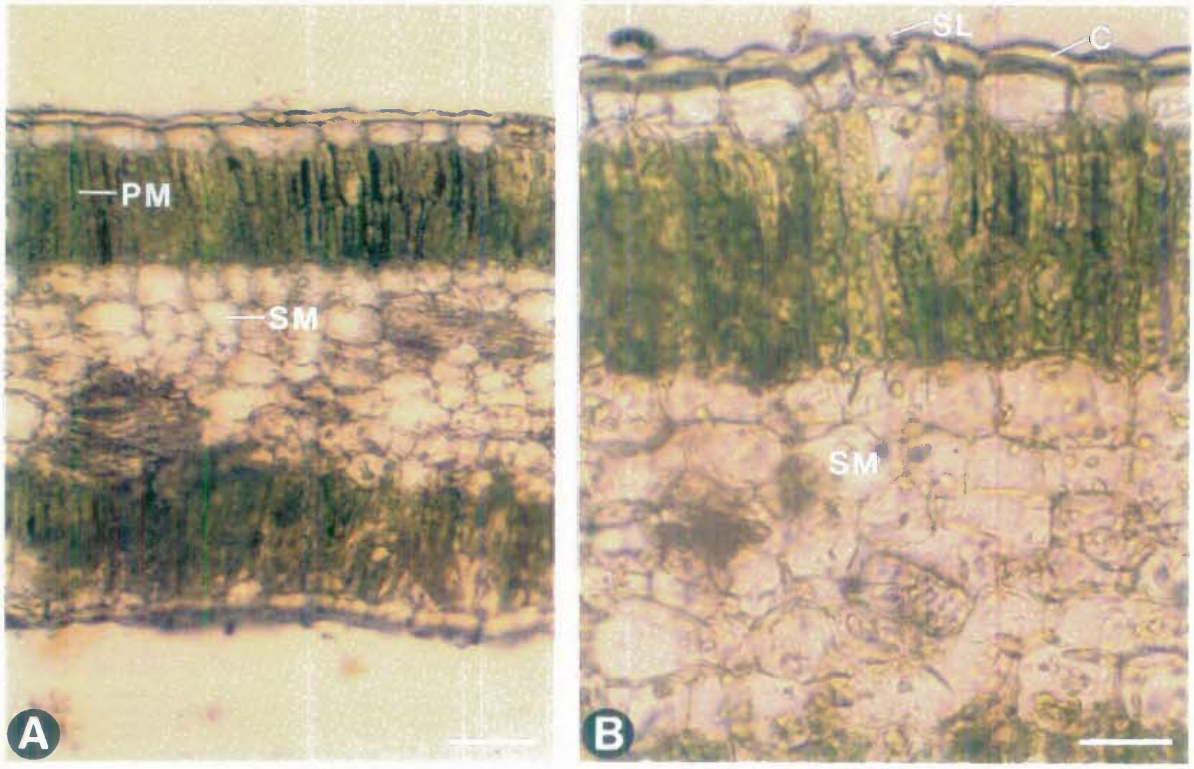


Photograph 8.2. Epidermal peel of the abaxial surface of an *A. amoena* phyllode showing a surface view of stomates. Note the paracytic arrangement of the subsidiary cells (S). G = guard cell. (bar = 25 μm)



Photograph 8.3. Transverse sections of *A. amoena* phyllodes, revealing some of the xeromorphic characteristics of this plant. (**A**) A higher proportion of palisade (PM) to spongy (SM) mesophyll (bar = 50 μm); (**B**) a thick cuticle (C), stomates protected by an outer stomatal ledge (SL), and few air spaces in the spongy mesophyll (SM) (bar = 25 μm).

Xylem fibres

The xylem fibres of *A. amoena* are li ɔriform with small, simple slit-like pits arranged irregularly along the radial walls (Photograph 8.4). Therefore, from an anatomical point of view, it is unlikely that such fibres play a sign ficant role in water conduction, especially as few pits are found between fibres and xylem vɛssels (Metcalfe and Chalk 1983). However, the role of fibres in water conduction is content ous (Zimmermann and Milburn 1982), as air bubbles have frequently been observed in xyler ı fibres (Wiegand 1906). Also, during conduit length determinations with indian ink (sectı on 9.2.2), the fibres appeared to be stained with indian ink, whereas freshly cut sections were a greenish colour. The examination of macerated material which had previously been infiltra ed with indian ink would confirm whether the ink was trapped by the fibre pits. Libriform wood fibres are common in woody dicots, particularly the Fabaceae (Eames and MacDaniels 1947).

Tyloses

Chattaway (1949) noted that specie with pit aperture diameters < 10 µm produced gums, and species with pit aperture diameters > 10 µm formed tyloses. However, tyloses have frequently been observed in *Acacia* (Photographs 8.5 - 8.7), a species with pit aperture diameters < 10 µm (Williamson 1989; and Photographs 8.1 c, d, e).

8.3.2 Searches for anatoı ical and microbial changes in *A. amoena*: A comparison of stems kept in:

(a) Distilled water; ınd

(b) Silver thiosulphate treatment, followed by citric acid and stabilised chlorine (SCC) vase solution

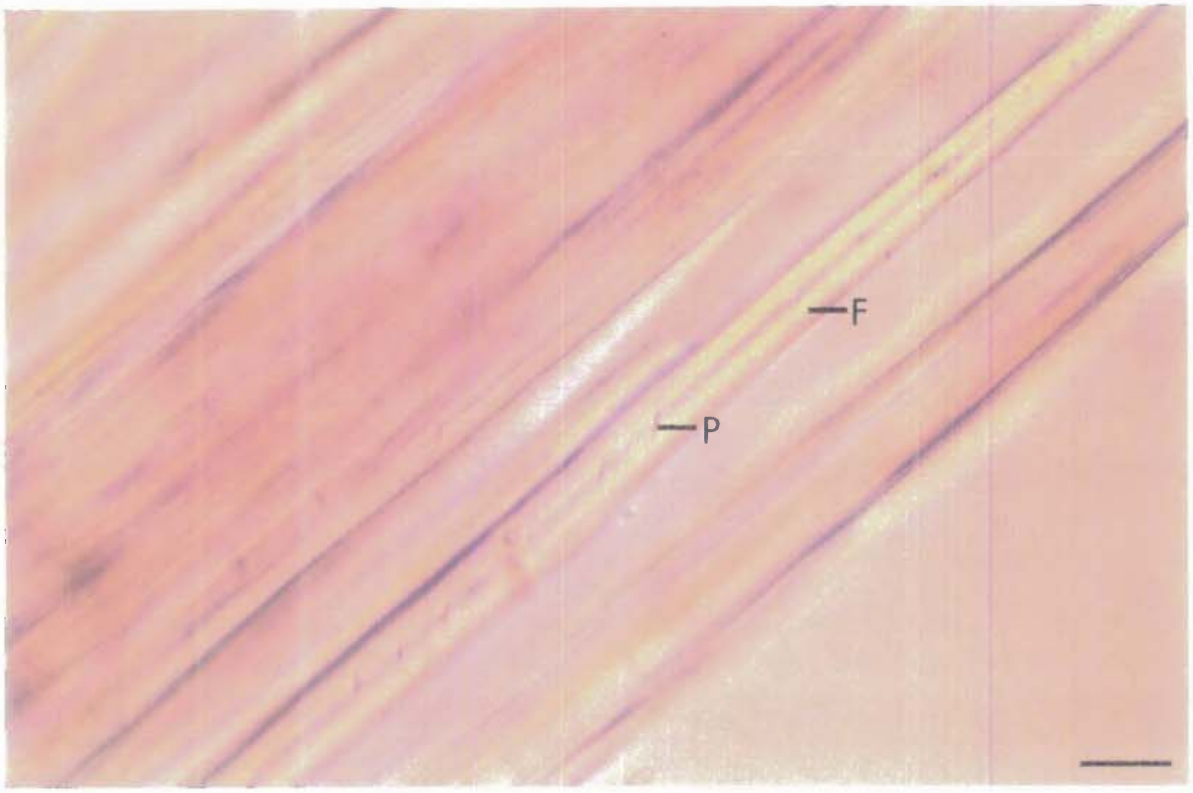
at days 1, 3 and 5 o vase life (using the CPD technique)

(a) Distilled water

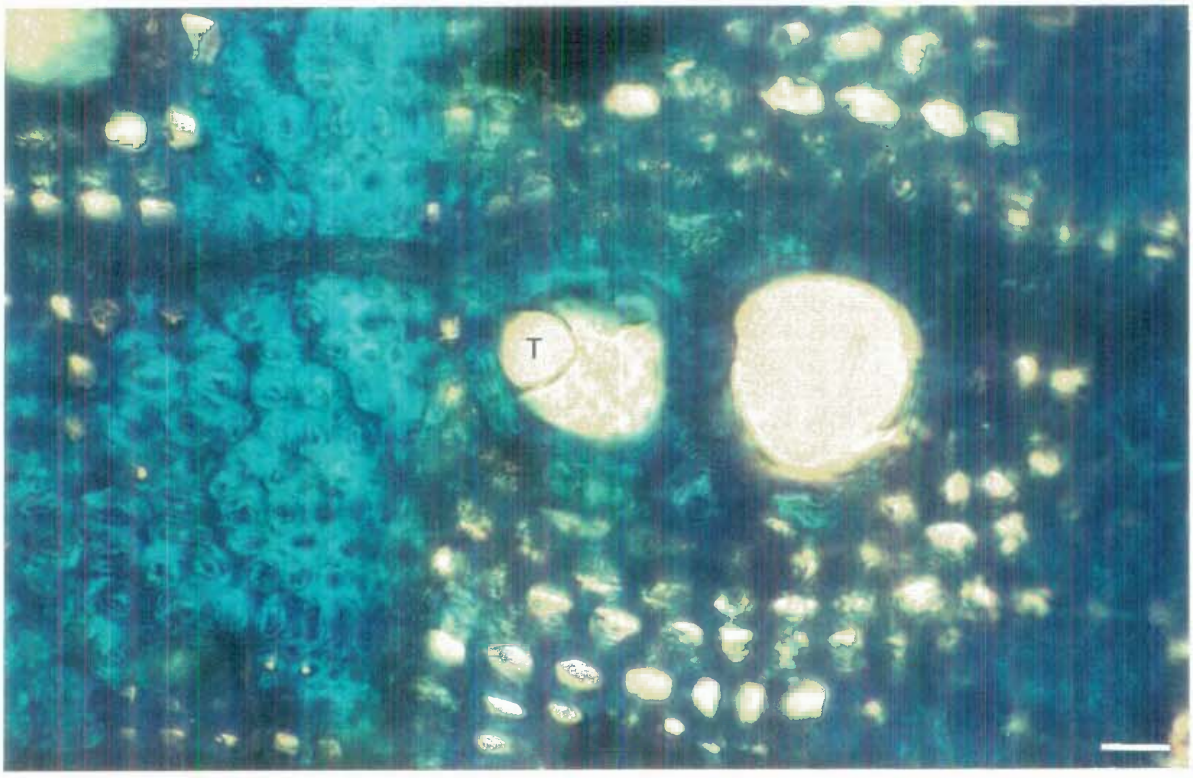
After 1 d of vase life, only a few bacteria were found in the cut stems (Photographs 8.8 a, b). The longitudinal section (LS) revealed that the pits were covered by fine fibrillar material (Photograph 8.8 a). Such fibrillar pit material had a similar appearance to vestured pits in the Myrtaceae, as shown under SEM (Dias-Leme *et al.* 1995).

After 3 d in distilled water, no bacteria could be seen, however, the sections were covered with loose material (Photographs 8.8 c, d), which might have obscured bacterial observation.

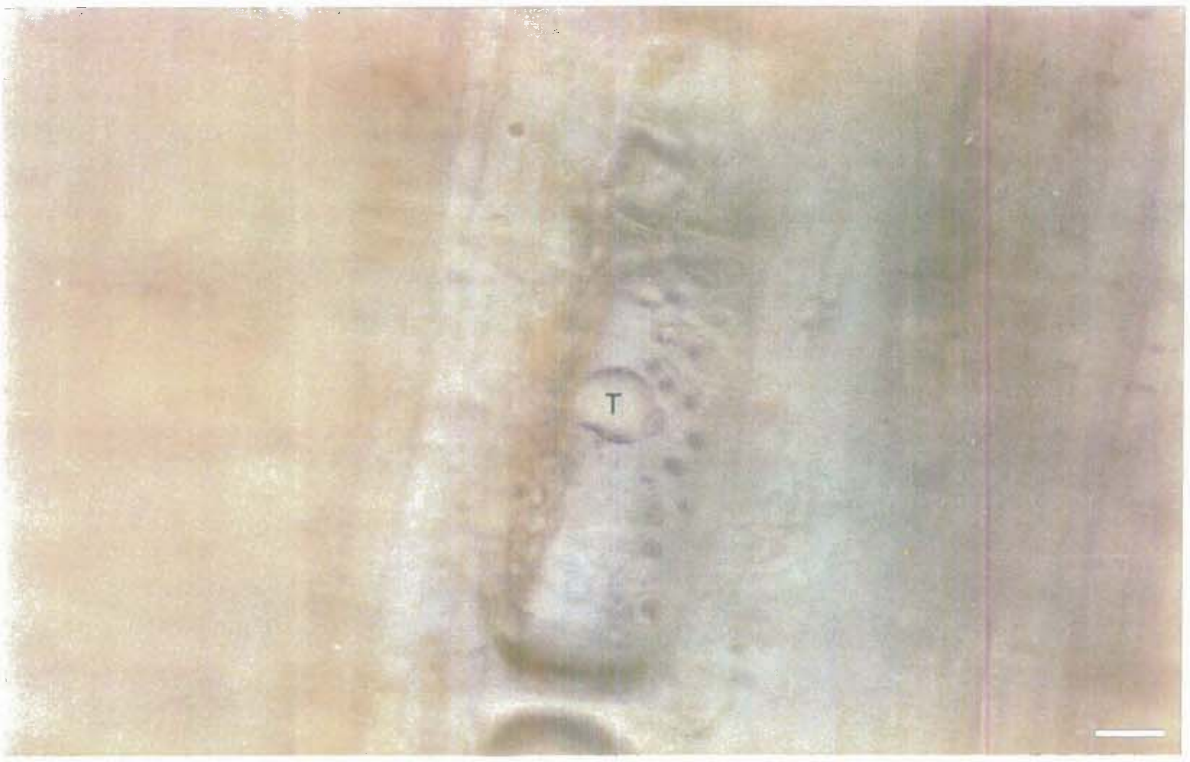
After 5 d, several bacteria were detected in the xylem of cut stems, both in LS (Photograph 8.8 e) and TS (Photograph 8.8 f). Additionally, the pits and inner walls of xylem conduits were covered with small deposits (Photograph 8.8 g). These deposits were similar in appearance to



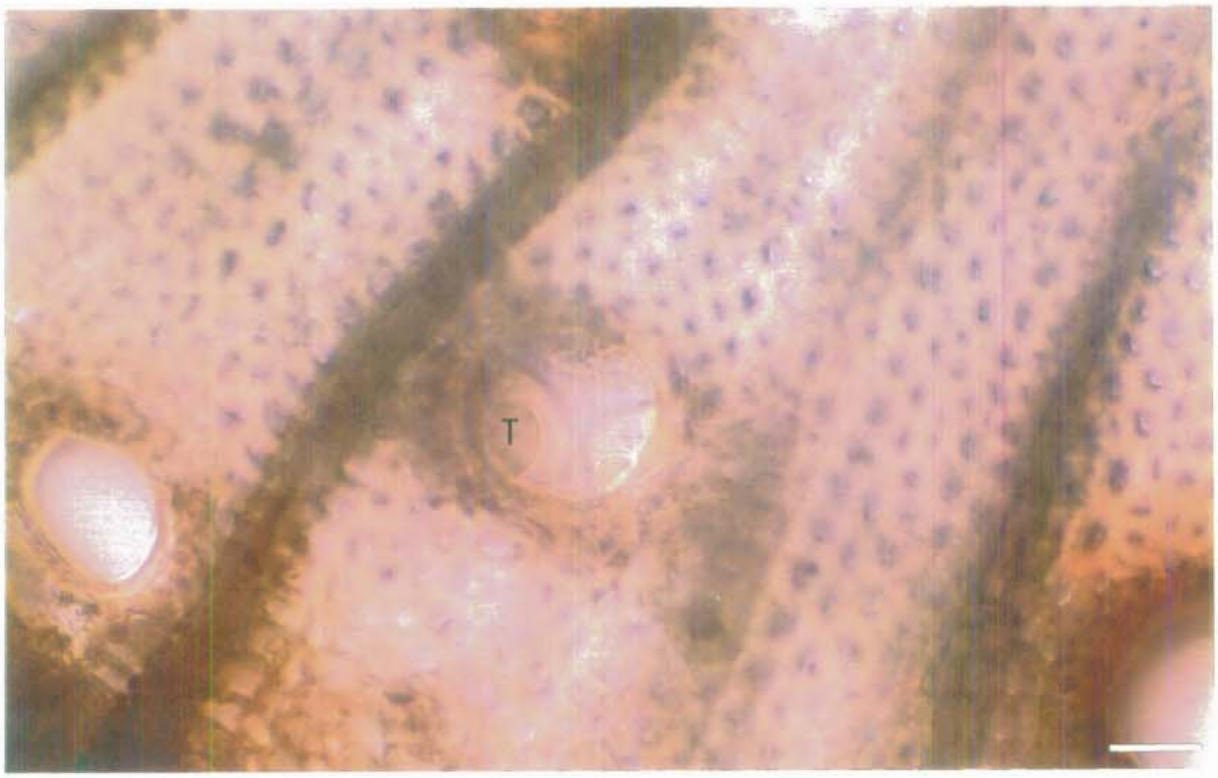
Photograph 8.4. Libriform wood fibres (F) of *A. amoena*. Note the small, simple pits (P). (bar = 25 µm)



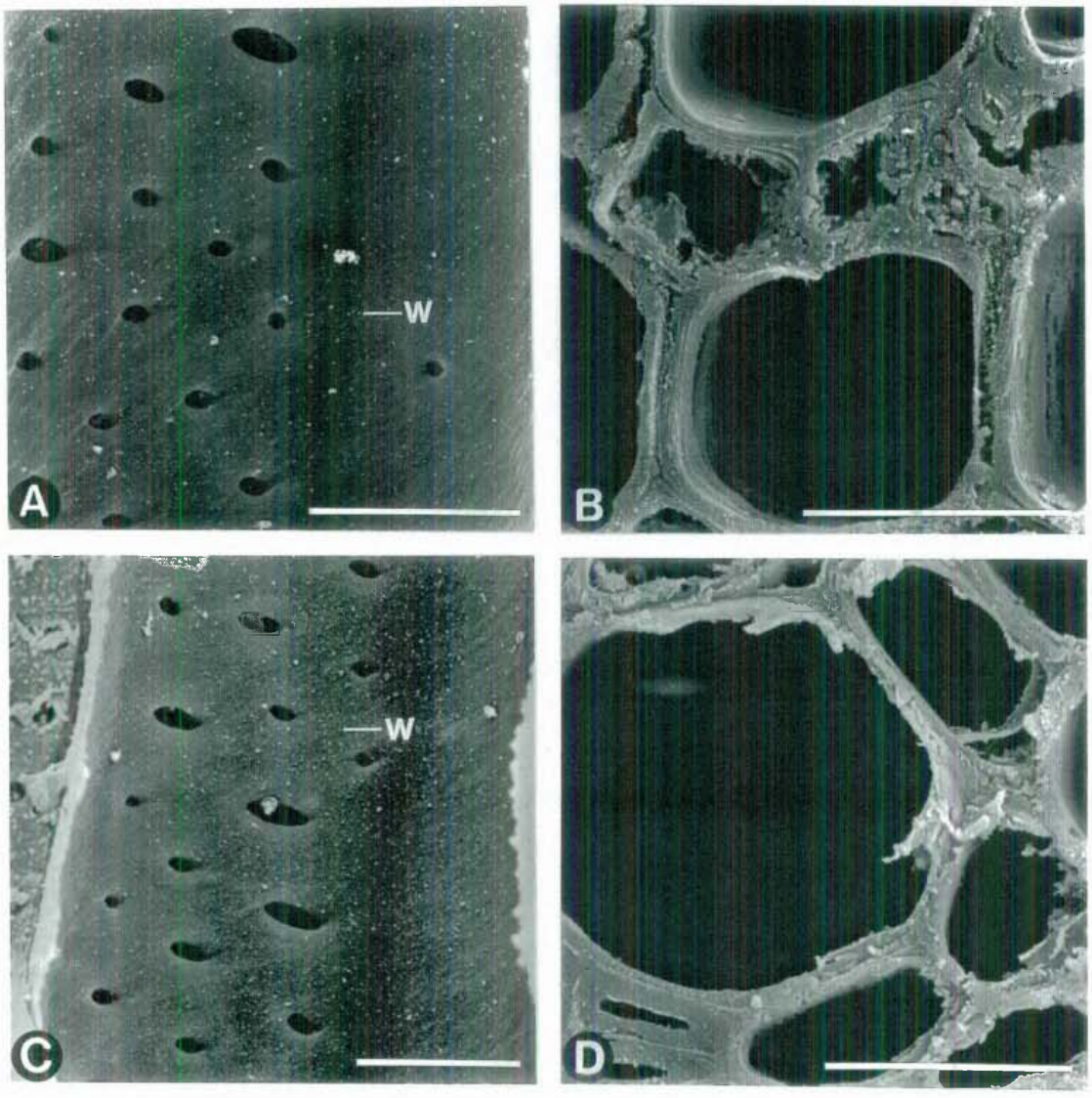
Photograph 8.5. Transverse hand cut section of an *A. floribunda* stem stained with toluidine blue, showing a tylose (T) partly occluding a xylem vessel (from Williamson 1989). (bar = 10 µm)



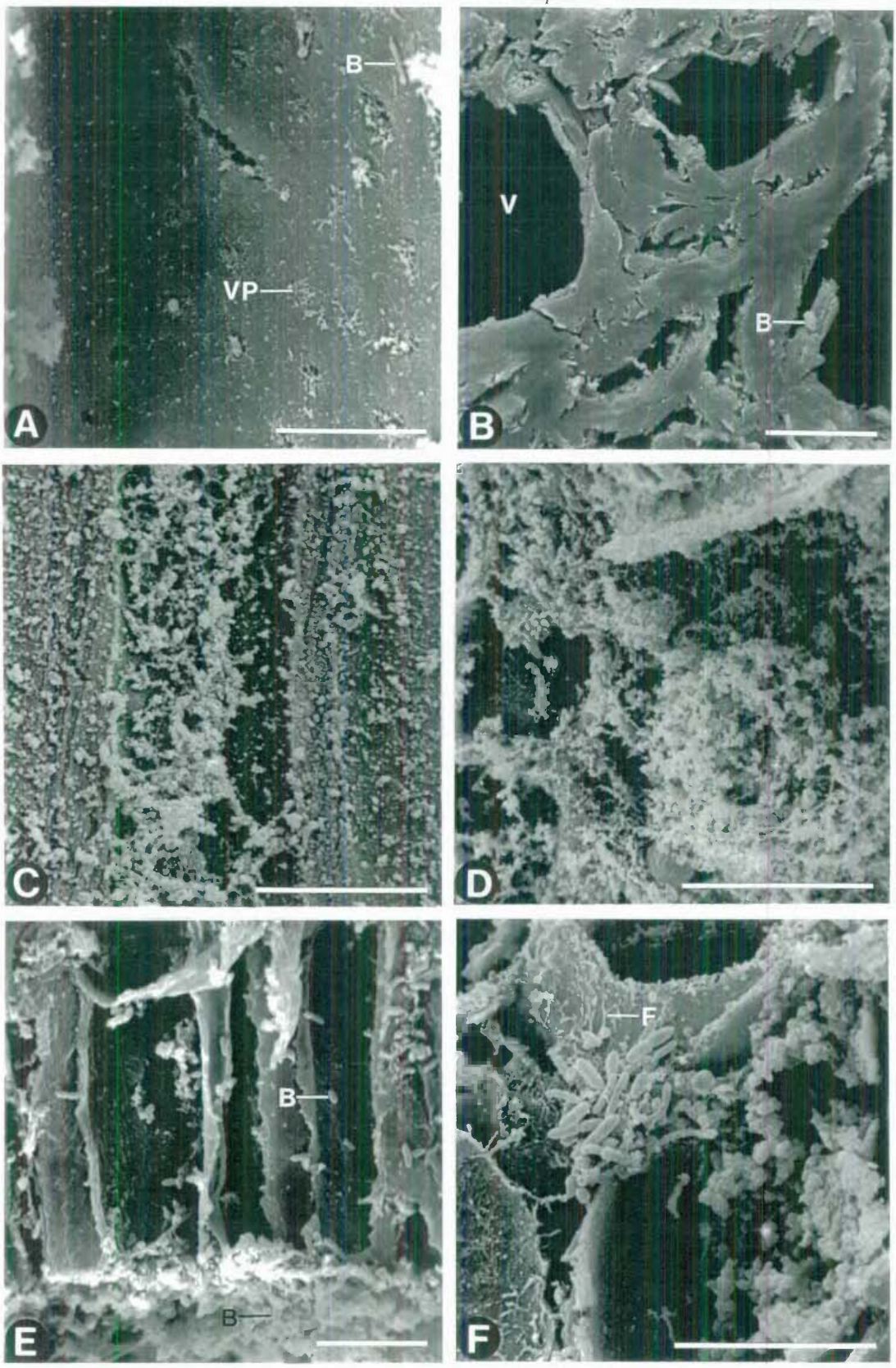
Photograph 8.6. Longitudinal hand cut section of an *A. floribunda* stem (unstained) showing a tylose (T) located within an air bubble in a xylem conduit. Several bordered pits are visible on the right hand side of the bubble (from Williamson 1989). (bar = 10 μm)



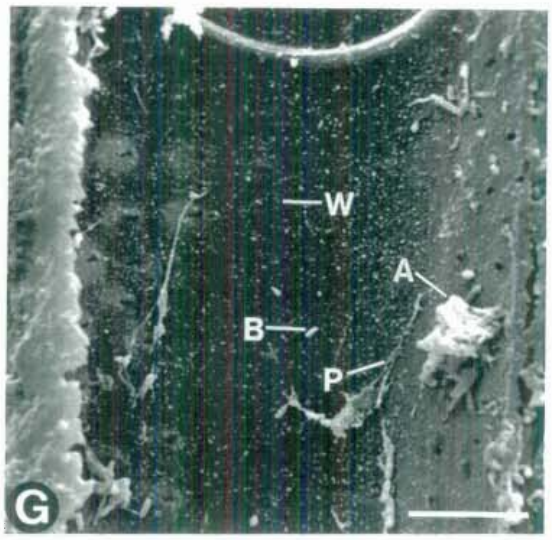
Photograph 8.7. Transverse hand cut section of an *A. amoena* stem (unstained) showing several tyloses (e.g. T) within the one xylem vessel. (bar = 25 μm)



Photograph 8.8a. SEM photomicrographs of the basal (cut) ends of freshly cut *A. amoena* and *R. hybrida* 'Sonia' stems (prepared by CPD) (bars = 10 μm). (**A**) Longitudinal section (LS) of *A. amoena* xylem conduit. The conduit is free of any bacteria. Note the presence of a warty layer (W). (**B**) Transverse section (TS) of xylem region of *A. amoena*. No bacteria or debris are present on the cut surface. (**C**) LS of *R. hybrida* 'Sonia' xylem conduit. The conduit is free of any bacteria. Note the presence of a warty layer (W). (**D**) TS of xylem region of *R. hybrida* 'Sonia'. No bacteria or debris are present on the cut surface.



Photograph 8.8. SEM photomicrographs of *A. amoena* cut stems kept in distilled water (prepared by CPD) (bars = 10 μm). (**A**) Longitudinal section (LS) of xylem conduit after 1 d. One (dividing) bacterium (B) is visible. Note the vestured pits (VP), which appear to be blocked by fibrillar material. (**B**) Transverse section (TS) of xylem region after 1 d showing three vessels (e.g. V) and one bacterium (B). (**C**) LS of xylem conduit after 3 d. No bacteria are visible, but the conduit wall is covered with loose material. (**D**) TS of cut surface of xylem after 3 d. The section is covered with loose material. (**E**) LS of xylem region after 5 d. Numerous bacteria (B) can be seen within the xylem conduits and also on the basal (TS) surface of the stem. Similar deposits to those seen in (**D**) above are also visible at the cut end. (**F**) TS of cut surface of xylem after 5 d. Several bacteria can be seen between xylem vessels. In addition, fibrillar (F) material can be seen.



(**G**) SEM photomicrograph showing an LS of an *A. amoena* xylem conduit after 5 d. The inner wall of the conduit is covered with small deposits resembling a warty layer (W). Some bacteria (B), material resembling dried protoplast (P) and "amorphous" deposits (A) can also be seen (bar = 10 μm).

the spread of vestured pits over the vessel surface, or to the morphologically similar warty layer (Wardrop *et al.* 1963; Butterfield and Meylan 1980).

(b) Silver thiosulphate/citric acid and stabilised chlorine (SCC) solution

After 1 d in SCC, only one bacterium was found within a xylem conduit (Photograph 8.9 a) and also at the basal end of the cut stem (Photograph 8.9 b). However, numerous small deposits and loose material were evident in both photographs.

After 3 d in SCC, some bacteria were evident in the xylem of cut stems. The LS (Photograph 8.9 c) reveals some bacteria within a xylem conduit, as well as some material which resembles dried protoplast (Meylan and Butterfield 1972a). The TS (Photograph 8.9 d) shows a similar picture to the LS, as some bacteria and material resembling dried protoplast, as well as numerous small deposits can be seen.

After 5 d in SCC, no increase in bacterial numbers was evident, as indicated by the LS (Photograph 8.9 e), which shows only one bacterium. However, numerous small deposits, resembling a warty layer, were seen on the inner conduit walls. The TS (Photograph 8.9 f) revealed that loose material formed clumps on the basal cut surface. No bacteria were evident in TS.

These observations on *Acacia* showed a lack of bacterial colonisation of the xylem over time in both distilled water and in SCC, and lead to the following experiments. Different methods of