Photograph 8.9. SEM photomicrographs of *A. amoena* cut stems kept in SCC (prepared by CPD). (**A**) LS of xylem conduit after 1 d. One bacterium is visible. Numerous small deposits can be seen (bar = 1 μm). (**B**) TS of basal end after 1 d, showing one bacterium (B) surrounded by loose material (bar = 10 μm). (**C**) LS of xylem conduit after 3 d. Some bacteria (B) are visible, as well as some material resembling dried protoplast (P) (bar = 10 μm). (**D**) TS of cut surface after 3 d. Two bacteria (B) are visible, as well as numerous small deposits and some material resembling dried protoplast (P) (bar = 10 μm). (**E**) LS of xylem conduit after 5 d showing one bacterium and numerous small deposits resembling a warty layer (W) (bar = 10 μm). (**F**) TS of basal end after 5 d. No bacteria are evident, but clumps of loose material can be seen (bar = 10 μm).

SEM specimen preparation with a genus commonly observed to contain bacteria, *Rosa*, were employed. The objective was to ascertain whether it was the preparation method, or the plant material used, that resulted in the low numbers of observed bacteria over time in *Acacia*.

8.3.3   Searches for anatomcal and microbial changes in *R. hybrida* 'Sonia': A comparison of stems kept in sterile tap water at days 1, 3 and 5 of vase life using the following preparation techniques:

(a)   Air Dried (AD);

(b)   Critical Point Dried (CPD);

(c)   Freeze Dried (FD); and

(d)   Cryo-SEM (or Frozen-Hydrated, FH)

(a)   Air dried (AD)

After 1 d in sterile tap water, bacteria were evident within xylem conduits of *Rosa* and at the cut basal end of the stem (Photographs 8.10 a, b). The cut surface was also covered by fine, granular material (Photograph 8.10 b).

After 3 d, the number of bacteria within the xylem conduits did not appear to have increased (Photograph 8.10 c), however, there was an increase in bacterial numbers at the cut surface (Photograph 8.10 d). The cut surface also appeared to have a thicker coating of granular material than was evident after 1 d. Fine, granular material, similar in appearance to the warty layer described by Butterfield and Meylan (1980), is visible on the inner surface of the xylem conduit (Photograph 8.10 c).

By day 5, bacteria were clearly visible in increased numbers within xylem conduits (Photograph 8.10 e). The inner wall was also covered with fine material resembling a warty layer. Some "amorphous" deposits are also evident in this section. In TS, the cut surface is shown to be covered by a thick granular layer (Photograph 8.10 f). Only a few bacteria are visible, although they may have been obscured by the thick granular layer, which appeared to increase over time.

(b)   Critical point dried (CPD)

After 1 d in sterile tap water, several bacteria were evident within the xylem, both in longitudinal (Photograph 8.11 a) and transverse (Photograph 8.11 b) sections.

After 3 d, no increase in bacterial numbers was evident within xylem conduits (Photograph 8.11 c). Evidence of a warty layer, and vestures around the pits can be seen in this photomicrograph. The TS shows an increase in bacterial numbers at the cut basal end (Photograph 8.11 d).

Photograph 8.10. SEM photomicrographs of *R. hyb ida* 'Son a' cut stems kept in sterile tap water and prepared for SEM observation by air drying (bars = 10 μm). (A) LS of xylem conduit after 1 d. Several bacteria (e.g. B) are visible. (B) TS of cut surface of xylem after 1 d. Some bacteria (B) are visible, but the surface is also covered by fine, granular material (G). (C) LS of xylem conduit after 3 d. One bacterium (B) can be seen. The inner surface of the conduit is covered by fine mater al with the appearance of a warty layer (W). (D) TS of cut surface of xylem after 3 d. Numerous bacteria (B) can be seen above a thick granular layer (G) covering the surface. (E) LS of xylem conduit after 5 d. Numerous bacteria (B) are visible around the pits (P). In addition, the inner wall is covered by a fine material with the appearance of a warty layer (W). Some "amorphous" deposits (A) are also visible. (F) TS of cut surface of xylem after 5 d. Some bacteria (B) are visible, but the surface is mainly coated by a thick granular layer (G).
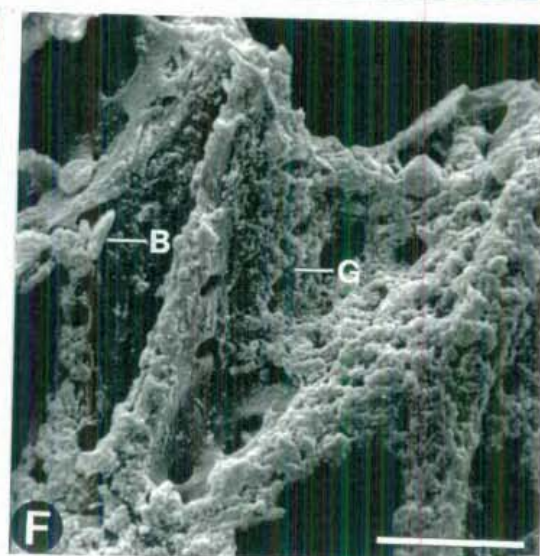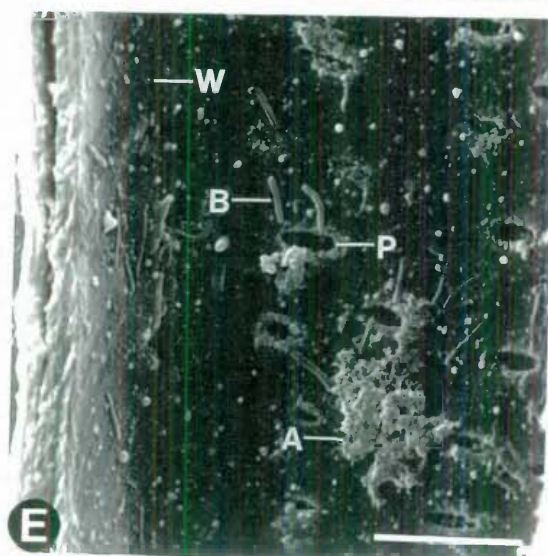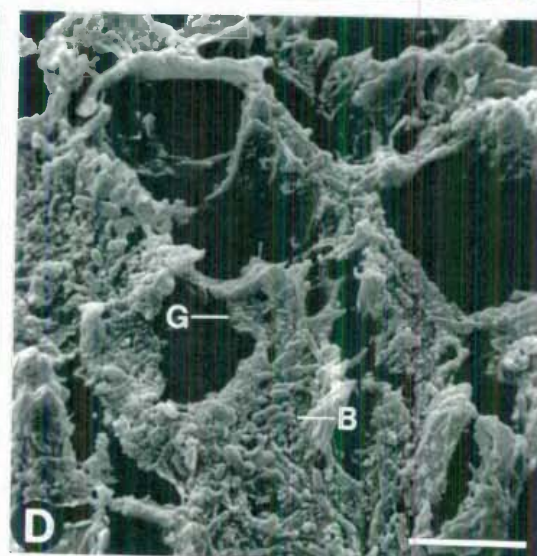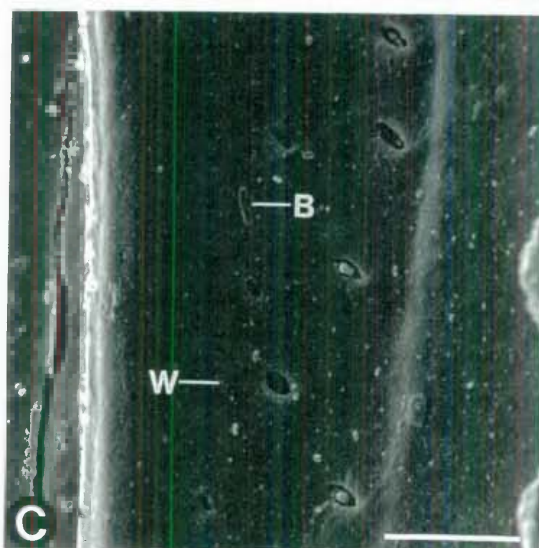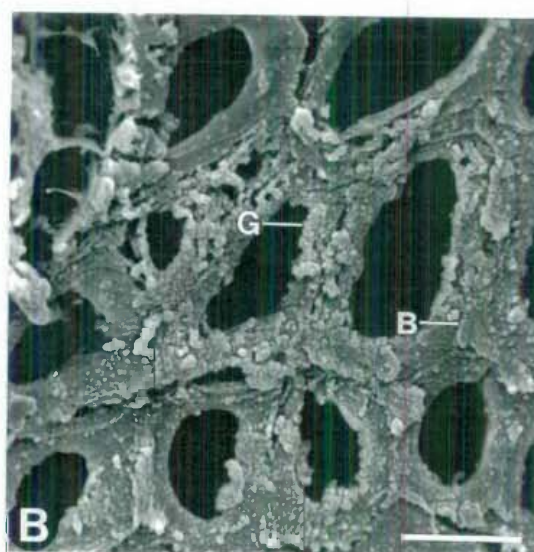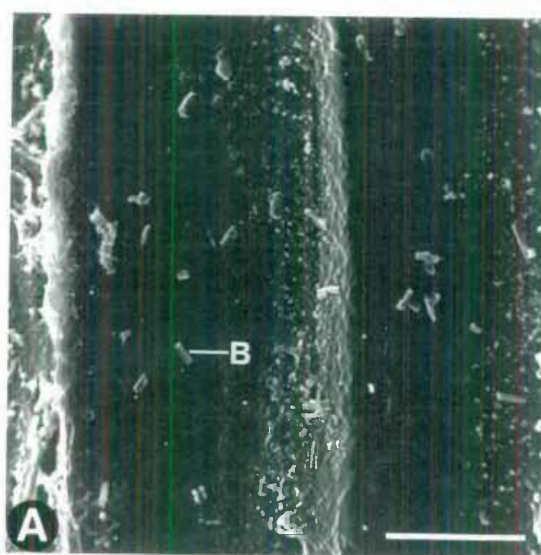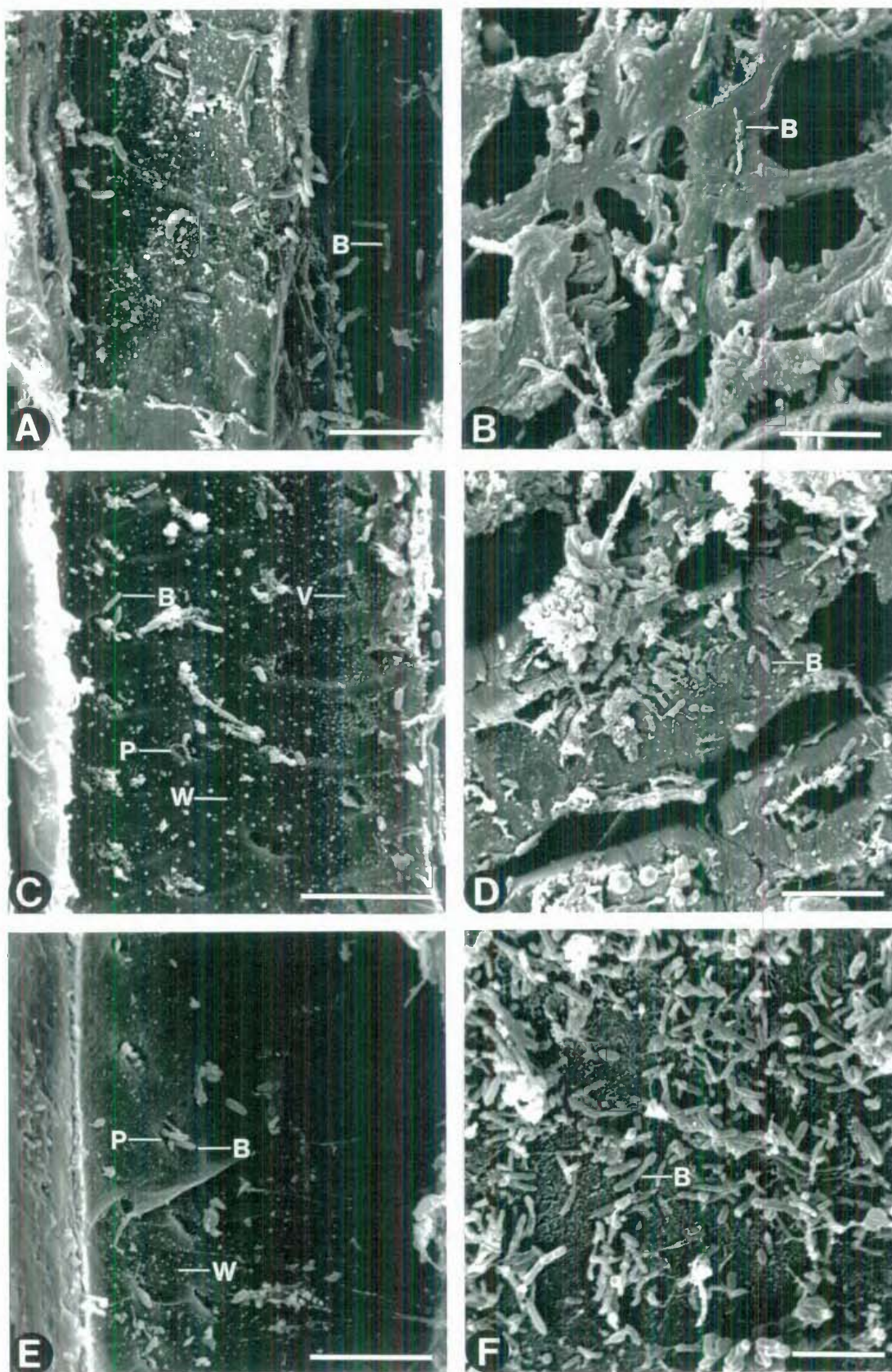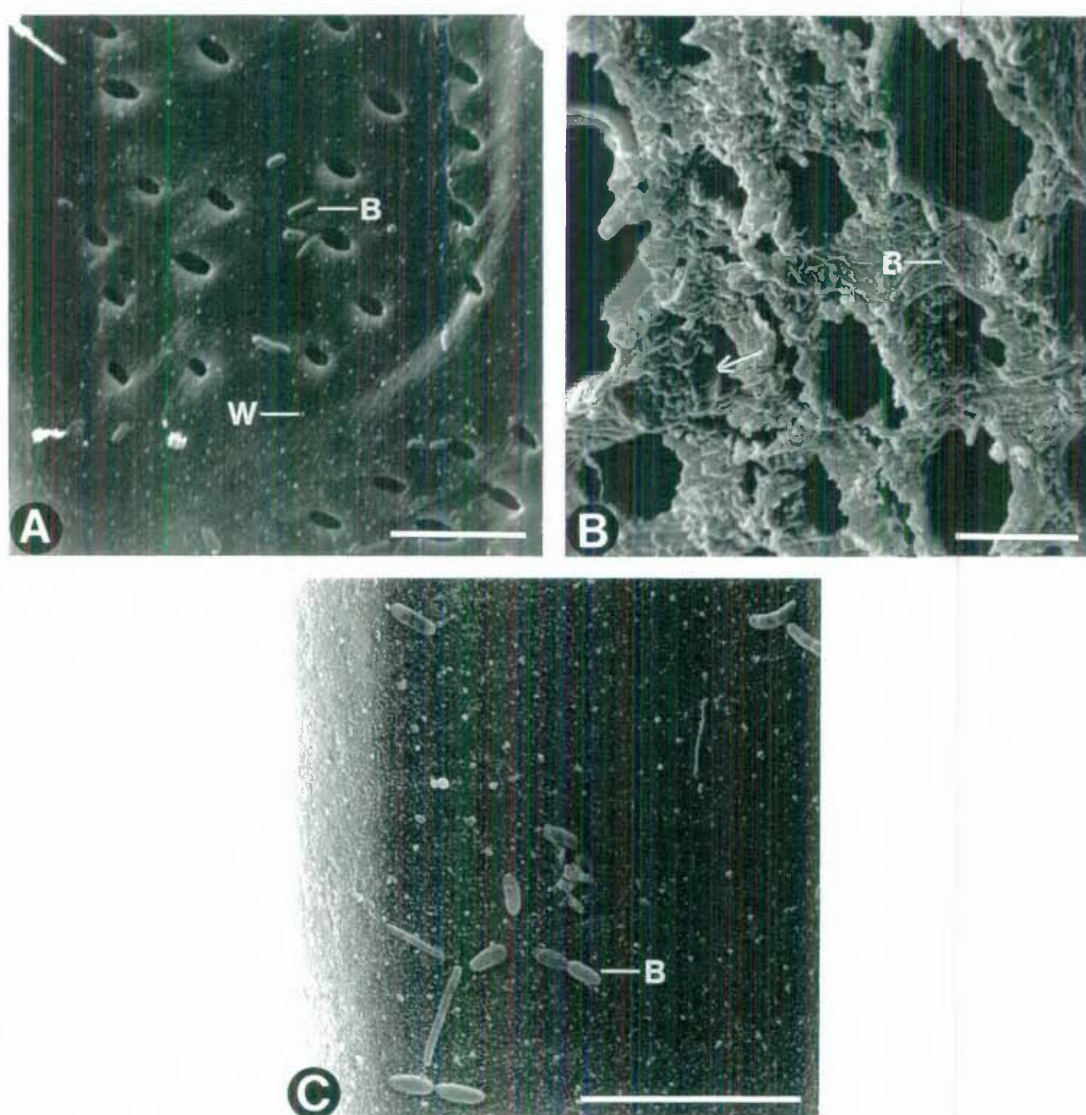
Photograph 8.11. SEM photomicrographs of *R. hybrida* 'Sonia' cut stems kept in sterile tap water and prepared for SEM observation by critical point drying (bars = 10 μm). (**A**) LS of xylem area after 1 d. Several bacteria (**B**) are visible. (**B**) TS of cut surface of xylem after 1 d. Several bacteria (**B**) are visible. (**C**) LS of xylem conduit after 3 d. Several bacteria (**B**) can be seen around the pits (**P**). Vestures (**V**) are also visible surrounding the pits. Fine material resembling a warty layer (**W**) is also evident. (**D**) TS of cut surface of xylem after 3 d. Several bacteria can be seen. (**E**) LS of xylem conduit after 5 d. Some bacteria (**B**) are blocking the pits (**P**). Fine material with the appearance of a warty layer (**W**) is also evident. (**F**) TS of cut surface of xylem after 5 d. Numerous bacteria (**B**) can be seen covering the cut surface.

By the fifth day, the LS shows similar numbers of bacteria to day 3, although some are blocking the pits (Photograph 8.11 e). However, the TS reveals a proliferation of bacteria at the cut basal surface after 5 d (Photograph 8.11 f).

(c)   Freeze dried (FD)

None of the day 1 specimens was examined under SEM because of a freeze dryer malfunction.

A few bacteria were visible within xylem conduits after 3 d in sterile tap water (Photograph 8.12 a). The fine, granular appearance of the warty layer covering the lumen surface is also evident in this photomicrograph. In TS, some bacteria can be seen on the cut basal surface (Photograph 8.12 b). The surface has a dehydrated, collapsed appearance.

Photograph 8.12. SEM photomicrographs of *R. hybrida* 'Sonia' cut stems kept in sterile tap water and prepared for SEM observation by freeze drying (bars = 10 µm). (**A**) LS of xylem conduit after 3 d. A few bacteria (**B**) are visible within the xylem conduit. Note the fine, granular appearance of the warty layer (**W**) covering the lumen surface. (**B**) TS of cut surface of xylem after 3 d. Some bacteria (**B**) are evident. Note the dehydrated, collapsed appearance of the cut surface (e.g. arrow). (**C**) LS of xylem conduit after 5 d. A few bacteria (**B**) are visible. Note the fine, granular appearance of the lumen surface.

After 5 d only a few bacteria were evident within the xylem conduits (Photograph 8.12 c). These bacteria appeared to belong to two different genera, because their shapes were very different. The lumen surface was covered by a fine, granular layer of material. (Due to an oversight, no photograph was taken of the TS at 5 d.)

(d)   Cryo-SEM

Observation of *R. hybrida* 'Sonia' tissue using this method was extremely unsatisfactory. The specimen could not be cryo coated or etched, as mentioned in section 8.2.3. It took more than 5 min from the time of specimen insertion into the SEM before any bacteria were located (at 2,000 × magnification). After a further 5 min, the image deteriorated rapidly and markedly, to the point where clear observation was no longer possible. The method was therefore abandoned before any photographic record was made[1].

8.4   DISCUSSION

The genus *Acacia* is "widespread and abundant, especially in arid and semi-arid areas" (Harden 1991). The anatomy of *A. amoena* (Photograph 8.3) reveals the xeromorphic nature of the species. A thick cuticle, stomates protected by an outer stomatal ledge, vascular bundles surrounded by sclerenchyma, and a high ratio of palisade to spongy mesophyll are all considered to be xeromorphic/xerophytic characteristics (Metcalfe and Chalk 1983). However, possession of these characters does not necessarily mean that such plants can withstand xeric environments. A true xerophyte may possess similar characters to a xeromorphic plant, but it is restricted to a dry locality (Metcalfe and Chalk 1983). A more accurate definition of a true xerophyte must be a plant that can survive in an extremely dry environment in which there is little available water (F. Went, pers comm. in Metcalfe and Chalk 1983) and high evaporation rates. For example, *Acacia aneura* (mulga) would be considered a true xerophyte because it can withstand xylem tensions of -12 MPa (Slatyer 1962), even though its distribution ranges from the arid zones of Australia (rainfall < 250 mm p.a.) to the wetter central western slopes of the upper Hunter Valley (32° S; 150° E in New South Wales (Harden 1991). Slatyer (1967) noted that *A. aneura* was an extremely drought-tolerant shrub. However, from a distribution sense, *A. amoena* must be considered a xeromorph. Its natural distribution is in mesophytic environments such as dry sclerophyll forests and woodlands and mountainous parts of the east coast of Australia. It does not grow farther west than the central western slopes of New South Wales (147° E) (Harden 1991).

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[1] After the experimental section of this thesis was completed, a new scanning electron microscope (Model JEOL JSM5800LV, Japan Electro Optics Laboratory Co. Ltd., Tokyo, Japan) was installed at UNE, Armidale, which had a low vacuum option fitted to the instrument. In this microscope, the specimen chamber is under less vacuum than the column, therefore hydrated, uncoated specimens can be examined without any image distortion, thus eliminating any artefacts produced during specimen drying and preparation.

Vestured pits (Photographs 8.1 c, d, e), which occur in many families possessing xeromorphic characteristics (e.g. Fabaceae, Myraceae, Proteaceae), are hypothesised to reduce the force acting on the pit membrane during e nbolisation when the pressure drops between two adjacent vessels. This is thought to reduce the risk of pit membrane rupture (Zweypfenning 1978). However, vestures can also be prese it on scalariform perforation plates and helical thickenings, which have no membranes (Butter ield and Meylan 1980). Furthermore, the occurrence of vestured pits is not limited to, or possessed by all, xerophytes (Zweypfenning 1978; Metcalfe and Chalk 1983), and therefore tleir function remains unclear. Vestured pits were also observed in *Rosa hybrida* 'Sonia' (Photographs 8.10 e, 8.11 c).

In many woods, especially of *Eucalyptus* spp. (Myrtaceae), the vestures of pits can spread out from the inner pit apertures to line the inner vessel walls (Butterfield and Meylan 1980). This was observed in *Acacia* (see, for example, Photographs 8.1 e, 8.8 g). Scurfield *et al.* (1970) noted that the spread of vestures beyond pit apertures was a usual feature in *Eucalyptus* and *Acacia*. In fact, they commented th it "in *Acacia* especially, this spreading makes it difficult to make any clear distinction between vestures and warts". The examination of plant cells by electron microscopy lead to the discovery of a "warty layer"[2] on the conduit walls of many gymnosperms, angiosperms (woody and herbaceous) and arborescent monocotyledons (Liese and Ledbetter 1963; Liese 1965). Warty layers were found in all species examined in the Fabaceae (Liese 1965). (The Rosaceae was not examined.) Vestured pits and warted internal vessel surfaces are common in angiosperms, and frequently, but not always, occur together. For example, they were both found in all but one of the 16 species (17 families) studied by Scurfield *et al.* (1970).

The warts appear to be localised thickenings of the cell wall (Wardrop and Davies 1962). The warty layer is thought to be formec during the last stage of cell differentiation. It consists of protoplast remnants, enclosed between the tonoplast and plasmalemma as the cytoplasm retracts and dries on the lumen surface (Wardrop and Davies 1962; Liese and Ledbetter 1963). When the tonoplast collapses on the plasmalemma, the remnants of cytoplasmic organelles are caught between the two membranes (Liese 1965). The chemical composition of warts is unknown, although lignin may be a component (Scurfield and Silva 1969). Warts and vestures are structurally and chemically similar (Scurfield and Silva 1970). Researchers have commented on the structural similarity of warts and vestures, particularly when the vestures extend beyond the pit aperture (Côté and Day 1962; Wardrop *et al.* 1963; Liese 1965). However, Schmid and

[2] Dutailly (1874) was the first to observe cribriform" pit membranes. He believed their "sieve-like" appearance was due to perforations in the pit membrar e through which protoplasmic connections occurred. However, Bailey (1933), in an admirable light microscopy study of 2,660 species in 979 genera and 152 families, was the first to recognise that the pit membrane was no: perforated. Rather, the sieve-like appearance was due to "minute outgrowths from the free surfaces of the econdary wall", which he described as "vestures". He noted that the outgrowths were not limited to the pit chambers, but also occurred on inner vessel wall surfaces, and used *Acacia* to illustrate the point. These outgrowths on inner vessel wall surfaces are now termed "warts" (Scurfield and Silva 1970).

Machado (1964) believed that the structures were different. They thought that the vestures formed first and were deposited directly on the cell wall outside the plasmalemma, whereas the warts arose later and were remnants of dead protoplast. However, as Scurfield and Silva (1970) pointed out, warts and vestures are both outgrowths of the secondary wall (Wardrop and Davies 1962). Therefore, Schmid and Machado's (1964) interpretation of the two structures as different is unlikely.

Liese (1965) noted that the distribution of warts on the lumen surface was regular, and local crowding was rare. However, vestures can spread from pit apertures into the vessel lumen and then "the vestured areas merge with those bearing warts" (Scurfield and Silva 1970), so that it is often impossible to distinguish between the two (Butterfield and Meylan 1980). Nevertheless, the fine materials which surrounded the pits of *R. hybrida* 'Sonia' (Photographs 8.10 e; 8.11 c) were vestures spreading from the pits onto the lumen surface, which graded into warts as the distance from the pits increased. Similar substances can be seen in published SEM photos of *Rosa* (Put and Clerkx 1988; Put and van der Meyden 1988; Put and Rombouts 1989), although they were not described as such by these authors. It is curious that the family Rosaceae is not considered to possess vestured pits (Metcalfe and Chalk 1950), and perhaps further study is warranted to unravel this anatomical inconsistency.

An attempt has been made to relate the absence of warts to phylogenetic advancement (Parham and Baird 1974). More primitive vessels (with scalariform perforations) exhibited warts, whereas more advanced vessels (with simple perforations) rarely had a warty layer. However, only 12 angiosperm species (six families) were examined, and therefore the conclusions are limited by the small sample size. Nevertheless, it can be said that the presence of warts would undoubtedly reduce the rate of water flow through xylem conduits.

The problem of the "amorphous" deposits seen in numerous SEM studies (e.g. Fujino *et al.* 1983; Put and Clerkx 1988; Put and van der Meyden 1988; Put and Rombouts 1989; van Doorn *et al.* 1991a, b; van Doorn, de Stigter, de Witte and Boekestein 1991) needs to be addressed. It is evident that some of these deposits should not have been described as "amorphous" (Put and Clerkx 1988; Put and van der Meyden 1988), because they are clearly warts[3]. The substance described by van Doorn *et al.* (1991a) as "amorphous" in maidenhair fern (*Adiantum raddianum* Presl.) tracheids near the pits, is in fact "obviously fibrillar [because] it pulls into fine webs across vessel pit apertures" (B. Butterfield, pers. comm. 1995). Such fibrillar material was first recorded by Witham (1833) and has been well documented over the years (see, for example, Duerden 1933; Evers 1951; Wesley and Kuyper 1951; Barghoorn and Scott 1958; Butterfield and Meylan 1972; Meylan and Butterfield 1972b; Butterfield and Meylan 1973; Butterfield and

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[3] Cronshaw (1960) made a similar error in referring to vestured pits as an "accumulation of amorphous material on the pit membrane" on the lumen side of vessels in *Eucalyptus regnans* (F. Muell.). The species is known for its vestured pits (Wardrop *et al.* 1963; Scurfield and Silva 1970).

Meylan 1982). The material has been known as fine vertical threads (Duerden 1933); "Williamson's striations" (Evers 1951); fimbrils (Barghoorn and Scott (1958) and microfibrillar webs (Meylan and Butterfield 1972b). The microfibrillar webs are undigested fragments of cellulose residues from the hydrolysi; of tracheid and vessel pit membranes and perforation plate partitions. They remain intact when they traverse the small openings of tracheid and vessel element pit membranes and perforation plates, particularly scalariform plates. However, they are not seen in wider openings as they are not considered strong enough to withstand the transpiration stream (Meylan and B itterfield 1972b; Butterfield and Meylan 1982). The term "amorphous" should, therefore, be t sed with care and not as a blanket term for any structures unknown to the author, when a little investigation of the literature may reveal the identity of currently 'unfashionable' structures.

Put and van der Meyden (1988) observed "mucoid or amorphic materials" in xylem vessels and around pits. As mentioned above, some of these materials are clearly warts (Fig. 6b), but others (Fig. 5a) appear to be more granular and of unknown origin. Meylan and Butterfield (1972a) observed similar granular material in fresh specimens of *Knightia excelsa* R. Br., a member of the Proteaceae. In *Knightia*, the granular material was the remnants of a granular partition material produced when the primary wall and middle lamella break down to form a perforation partition when a vessel member differentiates. Although the granular material observed in *Rosa* (Photographs 8.10 b, d, f; Put and v in der Meyden 1988) and *Acacia* (Photographs 8.8 c, d, f; 8.9 b, d, f) is of similar appearance to that seen in *Knightia* (Meylan and Butterfield (1972a), it is unlikely to be attributed to the same cause. Perhaps the granular material in cut flowers is synonymous with the breakdown of cellular substances that occurs during senescence. B. Butterfield (pers. comm. 1995) thought that the granular material seen in SEM photos of cut flowers may have been dried cell cytoplasm. He commented that it was often difficult to remove cellular material when attempting to obtain "clear" photographs of cell walls.

Exley *et al.* (1974) described a technique for removal of cytoplasmic debris from the surface of cut wood samples prior to SEM preparation. They found that soaking the wood in a 20% solution of sodium hypochlorite produced 'cleaner' specimens, thereby improving photographic appearance. Their untreated specimen of *Fuchsia* had a similar granular appearance to some of the SEM photos in this chapter (for example, Photographs 8.8 c, d, f; 8.9 b, d, f; 8.10 b, d, f). Thus, it is possible that some of the "mucoid or amorphic" materials described in some SEM studies (Fujino *et al.* 1983; Put and Rombouts 1989; van Doorn *et al.* 1991a, b; van Doorn, de Stigter, de Witte and Boekestein 1991) may in fact be cytoplasmic debris. It is uncertain what the effect of soaking bacteria in sodium hypochlorite might be, but it is worth considering this technique in order to distinguish between some "amorphous" or granular deposits and cytoplasmic debris. However, Fujino *et al.* (1983) observed similar "amorphous" material in maidenhair fronds kept in water for 2 d, yet no such deposits were found in freshly cut fronds

or those kept in $AgNO_3$, even after 15 d. This, they argued, was evidence against artefactual contamination.

Clark and Glagov (1976) thought that artefacts which appeared regularly could be mistaken for normal structures. In order to distinguish between artefacts from SEM preparation methods and true surface "contours", Meller *et al.* (1973) recommended examination of the same specimen under SEM and TEM. Such a comparative method may be appropriate in some cases so that the nature of some frequently observed ' amorphous", "jelly-like" and "mucoid" substances found in xylem conduits (Put and Rombouts 1989; van Doorn *et al.* 1991b; van Doorn, de Stigter, de Witte and Boekestein 1991) can be identified precisely. For example, van Doorn *et al.* (1991b) observed amorphous material accompanying bacteria in the xylem conduits of roses under both conventional SEM and cryo-SEM. In fact, there were more amorphous deposits under cryo-SEM than conventional SEM. The substance was thought to be bacterial slime, which dissolved during the dehydration process used in conventional SEM specimen preparation. Conversely, Schmitt and Liese (1993) observed ' amorphous" and fibrillar deposits under TEM after *Betula*, *Tilia*, *Fagus* and *Quercus* branches were wounded. These deposits appeared prior to blockage by suberin. The deposits were found in the pit chambers or around the pits in vessels or fibres. They considered such secretions to be an active resistance, initiated to protect against injury.

In this thesis, more bacteria were observed under SEM in *Rosa* than in *Acacia*. The reason for the difference is obscure. Thus, experiments were undertaken with *Rosa*, comparing different methods of SEM preparation. Initially, it was believed that the frequent changes of solution during the CPD method might have removed some of the bacteria in *Acacia*. However, this was shown not to be the case, because numerous bacteria were visible when this method was applied to *Rosa* (Photograph 8.11).

All methods of SEM specimen preparation (except cryo-SEM) were satisfactory in terms of image quality and ease of bacteria observation. The fastest preparation method was freeze drying, whereas CPD was the slowest. [It should be noted that the poorer photographic quality of some of the air dried specimens (Photograph 8.10 a, c,e) was due to difficulties encountered in setting the wave form monitor of the SEM prior to photography. This difficulty resulted in 'thin' negatives and a grey appearance when developed.] The specimen quality of air dried specimens was comparable to the other methods. In fact, several researchers have found OsO_4 vapour to provide the best images of all the chemical fixation methods (Falk *et al.* 1971). Exley *et al.* (1974) modestly reported that "good" results could be obtained by air drying, and Meylan and Butterfield (1972c) used air drying for most of the wood samples in their beautiful book of three-dimensional wood structure.

There was evidence that freeze drying produced some dehydration and collapse of the cut surface, although the bacteria were rot affected (Photograph 8.12 b). This was not seen in any other specimens, and was only apparent in TS. Quattlebaum and Carner (1980) found that freeze drying resulted in distorted, cracked and collapsed conidia and conidiophores. They attributed this to the expansion of water in hydrophilic structures during the quenching process.

The cryo-SEM method, using the equipment then available at UNE was unsatisfactory, probably because the unfixed material was not protected from electron bombardment and electrical charge accumulation, and so deteriorated rapidly. The frozen specimen may also have been warmed by the electron beam (Turner and Smith 1974). To avoid electron beam damage, Turner and Smith (1974) recommended that the specimen be focused quickly and, preferably, slightly to one side of the area to be photographed. Although Falk *et al.* (1971) found that fresh, unfixed shoot tips of *Tropaeolum majus* viewed under conventional SEM produced excellent images, their photographs were always made within 5 min of specimen insertion and under very low magnification (160 x). However, bacterial colonies are not always readily apparent in xylem tissue, and a magnification of approximately 2,000 x is necessary to view and photograph them clearly. By the time bacteria were found, the image had deteriorated. Falk *et al.* (1971) noted that high magnification (level not stated) resulted in large amounts of energy being directed towards a small area of the specimen, which resulted in specimen movement and shrinkage. Charging damage also occurred when the electron beam was concentrated under high magnifications (Turner and Smith 1974). Problems with specimen charging of fresh, uncoated, frozen plant material examined on a cold stage were encountered by Parsons *et al.* (1974).

No anatomical changes were observed over the 5 d period in either *Acacia* or *Rosa* for any SEM preparation method. SEM will only reveal visible blockages, such as those caused by the presence of micro-organisms or other blocking materials. It will not show evidence of conduits that have become blocked or non-conducting via embolisation. Rasmussen and Carpenter (1974) noted that cut roses, placed in solution, exhibited severe wilting after 24 h, but they could not detect any xylem conduit blockage under SEM. Embolised conduits are the likely explanation for Rasmussen and Carpenter's (1974) SEM findings. It is now accepted that vascular blockage can occur from several causes, i.e. physiological blockage (cavitation and subsequent embolisation of xylem conduits; the formation of vascular plugs from various causes (e.g. wounding); and microbiological blockage (either physically or through production of substances, e.g. ethylene or enzymes) (van Doorn 1989; Put 1991). Therefore, SEM searches for xylem blockages are limited to the visible, physical causes, and it is perhaps not even sensitive enough to detect all of those.

Whilst SEM can provide excellent visual images, an operator may 'see and look for what they want to see', possibly by-passing (i.e. not photographing) many areas in the examination of a

specimen. This subjective bias may be an unconscious characteristic of SEM studies. Selectivity may be appropriate in some instances, e.g. phytopathological infection studies examining the growth of germ tubes and formation of appressoria and penetration pegs for penetration into the host epidermis However, when studying bacterial occlusion of xylem vessels, it is likely that the method is not sufficiently quantitative. All the microbial SEM studies seen by the author (including those described in this chapter) show the presence or absence of micro-organisms in a non-quantitative manner. Theoretically, it would be possible to quantify SEM studies by counting the number of micro-organisms in random fields of view, as is done under light microscopy for plant pathological studies. Nevertheless, Put and Clerkx (1988) thought that SEM was not sensitive enough to detect low numbers of micro-organisms in xylem, and advocated the plate count method (described in sections 6.2.4 and 6.2.5) for an accurate representation of microbial numbers.