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Scanning electron microscopy of hydrated and desiccated mature somatic embryos and zygotic embryos of white spruce (*Picea glauca* [Moench] Voss.)

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Summary. Four scanning electron microscope techniques for preparing somatic and zygotic embryos of white spruce (*Picea glauca* [Moench] Voss.) were compared. Direct sputter coating without critical point drying worked well for desiccated embryos while conventional methods using chemical fixation were appropriate for hydrated somatic embryos. Low temperature scanning electron microscopy and plastic replicas provided excellent specimens of all embryos studied. Plastic replicas were used to document cotyledon formation and growth during maturation of somatic embryos. Apart from some differences in embryo size, orientation of cotyledons and surface wrinkling, the general morphology of mature somatic embryos of white spruce was very similar to zygotic embyros at a similar stage of development.

Key words: *Picea glauca* - Scanning electron microscope methods - Somatic embyros - Maturation - Desiccation

Introduction

Somatic embryogenesis, the formation of embryos from somatic cells, has been used to propagate a wide range of angiosperms since the method was introduced for tissue cultures in 1958 (reviewed by Ammirato, 1983). This technology was first reported for conifers in 1985 and since then has been described for more than 20 species representing 6 genera of conifers (Attree and Fowke 1993).

Maturation methods have recently been developed which yield high quality somatic embryos of white spruce with increased levels of storage lipids and proteins (Attree et al., 1991,1992; Misra et al., 1993). Such embryos can be desiccated to moisture levels below that of zygotic embryos and stored for prolonged periods before being imbibed and germinated to yield plantlets at high frequencies.

Light microscopy and conventional transmission electron microscopy (TEM) have been used to compare the cytology of mature hydrated somatic embryos and imbibed somatic embryos of white spruce following desiccation with that of mature imbibed zygotic embryos dissected from seeds (Attree et al., 1992; Misra et al., 1993). Somatic embryos contained storage reserves in the form of lipid and protein bodies comparable to those of mature zygotic embryos. The external morphology of hydrated and desiccated conifer embryos has been examined by light microscopy but not by scanning electron microscopy (SEM). Ultrastructural studies (TEM, SEM) have shown that most somatic embryos of angiosperms are morphologically and developmentally quite different from zygotic embryos (e.g. Vasil and Vasil, 1981; Burwale et al., 1986; Xu and Bewley, 1992; Brisibe et al., 1993). Similar SEM studies comparing conifer somatic and zygotic embryos have not been reported.

The aims of this study were first to examine by SEM the external morphology of hydrated and desiccated somatic embryos of white spruce to look for evidence of damage during desiccation and to compare their morphology to that of dry zygotic embryos dissected from seeds. Secondly, to undertake this study it was necessary to identify the best methods for preparing hydrated and desiccated embryos of white spruce for examination by SEM.

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Materials and Methods

Plant material - Embryogenic suspension cultures of white spruce (*Picea glauca* [Moench] Voss.) were established and maintained as described previously (Hakman and Fowke, 1987; Attree et al., 1989). Immature embryos were matured and subsequently desiccated according to published methods (Attree et al., 1991, 1992). Hydrated and desiccated mature somatic embryos as well as zygotic embryos dissected from dry seeds (Saskatchewan Government, Forestry Branch, Prince Albert Nursery) were prepared for examination in the SEM by the following methods.

Preparation Method 1: No fixation, direct sputter coating. Hydrated and desiccated somatic embryos as well as desiccated zygotic embryos were mounted on SEM stubs with adhesion transfer tape (J.B. EM Services, Dorval, Quebec), sputter coated with gold in an Edwards Sputter Coater, Model S150B, and then examined in a Philips 505 Scanning Electron Microscope.

Preparation Method 2: Chemical fixation, critical point drying. Hydrated somatic embryos were processed according to the methods of Fowke (1994). Briefly, embryos were fixed sequentially in 1% and 3% glutaraldehyde in 0.025 M phosphate buffer, pH 6.8, for 1 h each at room temperature. They were washed in buffer, placed on ice, then postfixed overnight in 1% osmium tetroxide in the same buffer at 0°C. Embryos were washed in distilled water, slowly dehydrated at 0°C in acetone, critical point dried in a Polaron E3000 Critical Point Dryer and then mounted and sputter coated with gold as above. Desiccated somatic and zygotic embryos were not prepared by this method.

Preparation Method 3: Low temperature SEM (LTSEM). Desiccated somatic and zygotic embryos and hydrated somatic embryos were processed according to published methods (Robards and Crosby, 1979; Attree and Sheffield, 1984) and viewed at -180°C using a Hexland cryosystem on a JEOL 840A SEM. Briefly, embryos were mounted in various orientations on chucks using a mixture of Tissue-tec and conducting carbon cement (Leitz). Embryos were flash frozen in liquid N_2 slush (-230°C) in an atmosphere of dry argon gas, inserted into the cryo SEM and heated slowly to -80°C to sublimate surface ice. Embryos were then returned to the coating chamber, cooled to -180°C and coated with gold. They were then reinserted into the cold stage of the SEM at -180°C and examined.

Preparation Method 4: Plastic replicas. Plastic replicas of mature desiccated somatic, zygotic and hydrated somatic embryos as well as somatic embryos at different stages of maturation were prepared according to the methods of Williams and Green (1988). Briefly, moulds were made using a mixture containing equal quantities of base paste and catalyst paste of GC Exaflex vinyl silicone dental impression material, injection type (Sinclair Dental, Saskatoon, Canada). Moulds were formed by either immersing the embryo in the mixture or coating the embryo with the freshly mixed silicone and allowing the mixture to harden. Moulds were then gently removed from the embryos. Casts were prepared by filling the moulds with freshly mixed CONAP Easy Epoxy (CONAP Inc., Allegany, NY) which hardens slowly at room temperature and is not viscous thus minimizing bubble formation. Bubbles were removed with a fine beaded glass needle drawn from a pasteur pipette. The epoxy was then baked at 60°C for 2 h. The hardened epoxy caste was removed from the mold, mounted on a stub, sputter coated with gold and then examined in the SEM.

Results and Discussion

When transferred to maturation medium containing abscisic acid and high molecular weight polyethylene glycol, immature suspension cultured somatic embryos matured over a 4-8 week period (Attree et al., 1991). Figure 1 shows a developmental sequence of somatic embryos from immature to mature stages. Embryos matured in this manner were desiccation tolerant and could be dried to low moisture levels (Attree et al., 1991). They were shrivelled (Fig. 2) and exhibited an opaque yellow colour. Figure 3 illustrates mature zygotic embryos dissected from dry white spruce seeds for comparison. The somatic embryos were larger in diameter than zygotic embryos and had an open array of cotyledons unlike the tightly folded cotyledons of the zygotic embryos. Both of these features of somatic embryos are likely due to the absence of the megagametophyte which would probably constrict the size of the embryo and determine the alignment of the cotyledons. Otherwise, the two embryos were morphologically quite similar. Transmission electron microscope studies have also demonstrated an overall similarity between white spruce somatic and zygotic embryos in terms of internal cell structure and distribution of storage reserves (Attree et al., 1992; Misra et al., 1993). In contrast, somatic embryos of angiosperms typically differed markedly in morphology from their zygotic counterparts (e.g. Xu and Bewley, 1992; Brisibe et al. 1993). The altered morphology of somatic embryos is likely due to the tissue culture environment where access to growth hormones and nutrients would not be regulated as they are in the

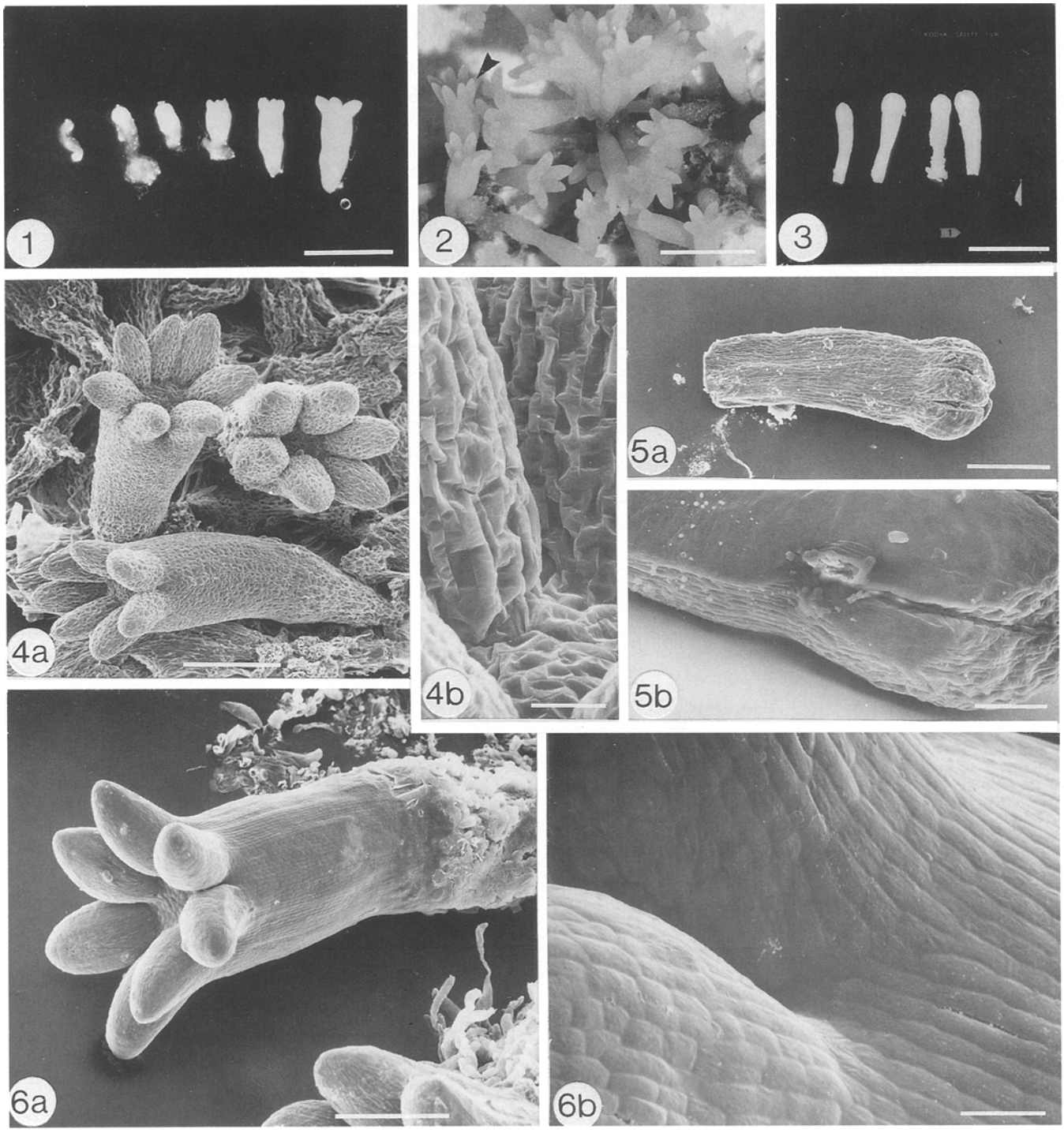

Fig. 1. Developmental stages of white spruce somatic embryos from immature to mature, bar = 2 mm. **Fig. 2**, Desiccated somatic embryos. Note the fused cotyledons (arrow), bar = 2 mm. **Fig. 3**, Zygotic embryos dissected from dry seeds, bar = 2 mm. **Fig. 4a**. Desiccated somatic embryos prepared for SEM by sputter coating alone (method 1), Note the uniformly wrinkled surface, bar = 0.5 mm. **Fig. 4b**. Wrinkled cells on one of the embryos in 4a, bar = 50 μm. **Fig. 5a**. Zygotic embryo from dry seed prepared for SEM by sputter coating alone (method 1). The cotyledons are tightly folded, bar = 0.5 mm. **Fig. 5b**. Smooth side of same embryo as in 5a, bar = 0.1 mm. **Fig. 6a**. Hydrated somatic embryo prepared for SEM using chemical fixation and critical point drying (method 2), bar = 0.5 mm. **Fig. 6b**. Cell detail from same embryo as in 6a, bar = 50 μm.

developing seed.

Comparison of preparative methods for SEM. All SEM preparative methods evaluated in this study proved useful for examining the external morphology of somatic and zygotic embryos. Some were suitable for hydrated embryos and others for desiccated embryos. The methods also varied considerably in equipment required and level of complexity. The suitability of the four methods for preserving white spruce embryos for SEM examination is summarized in Table 1.

Table 1. Quality of specimen preservation using different methods for preparing hydrated and desiccated embryos of white spruce for SEM.

Method	Embryo Type: Hydrated Somatic	Embryo Type: Desiccated Somatic & Zygotic
No fix, direct sputter coat	poor	good
Chemical fix, CP dry	good	----
Low temperature SEM	good	good
Plastic replicas	good	good

Preparation Method 1: No fixation, direct sputter coating. Direct sputter coating is the simplest of the methods evaluated. It requires access to a sputter coating unit or alternatively a vacuum evaporator for coating specimens with gold. This method of preparation worked well for desiccated somatic and zygotic embryos of white spruce (Figs. 4,5) but was unsuitable for hydrated specimens which collapsed under vacuum during the gold coating process and in the SEM (not shown).

Figure 4 shows the surface structure of three somatic embryos prepared by this technique. Apical meristems surrounded by open cotyledons were clearly visible. The surface of these embryos was uniformly shrivelled due to severe wrinkling of the cells. Direct evacuation of desiccated embryos in the sputter coater and then the SEM did not appear to cause further wrinkling of the embryo surface (c.f. methods 3 & 4 below). This is likely due to the fact that desiccated somatic embryos contain less moisture than zygotic embryos (Attree et al., 1991).

The apical meristem region of mature zygotic embryos dissected from dry seed was obscured by the tightly packed cotyledons (Fig. 5a). Much of the surface of dry zygotic embryos was also shrivelled but the cells appeared less wrinkled than those of somatic embryos; most zygotic embryos also exhibited large smooth patches (Fig. 5b), often covering one side of the embryo. These large smooth surfaces seem to be the result of the curved position of the embryo in the seed; the convex side in contact with the megagametophyte was generally smooth and the concave side wrinkled. The less extensive wrinkling of surface cells on the concave surface of zygotic embryos compared to somatic embryos is probably a result of the much slower drying process during seed development and the fact that zygotic embryos are known to have a higher density (% dry weight) of storage reserves (Attree et al., 1992).

Preparation Method 2: Chemical fixation, critical point drying. This method of preparation requires access to a critical point drier as well as a gold coating unit. Hydrated somatic embryos fixed with conventional chemical fixatives, dehydrated, critical point dried and then sputter coated were well preserved (Fig. 6). The embryos appeared turgid, cell surfaces were smooth and little or no cell shrinkage was detectable. Cell patterns were more clearly visible here than when observed by low temperature SEM (method 3 below). The apparent increased surface detail may result from the removal of surface waxes by the solvents used for dehydration and critical point drying. The external morphology of desiccated somatic and zygotic embryos could not be retained using this method since conventional fixation hydrates the specimens.

Preparation Method 3: Low temperature SEM (LTSEM). Both hydrated and desiccated mature somatic embryos as well as dry zygotic embryos (not illustrated) of white spruce were well preserved when rapidly frozen (without chemical fixation), transferred to the cold stage of a SEM then coated and examined while still frozen (Figs 7,8). This method should give an accurate representation of the natural state of both dry and hydrated embryos since they were observed without exposure to chemicals or further drying. The hydrated embryos appeared turgid with smooth surfaces (Fig. 7) and were similar to those prepared by conventional fixation and critical point drying (cf method 2). Desiccated embryos were shrivelled and were characterized by wrinkled cells (Fig. 8) as observed with direct sputter coating (cf method 1). Fine detail at cell surfaces of both types of embryos was more difficult to detect, possibly due to the retention of waxes on the cuticle or perhaps ice. While preservation was excellent for both types of embryos, the level of contrast achieved with this microscope did not match that obtained with the standard SEM due to the low voltage used to eliminate charging effects.

A major disadvantage of this technique is the need for sophisticated costly SEM equipment. The method is also subject to a number of freezing artifacts

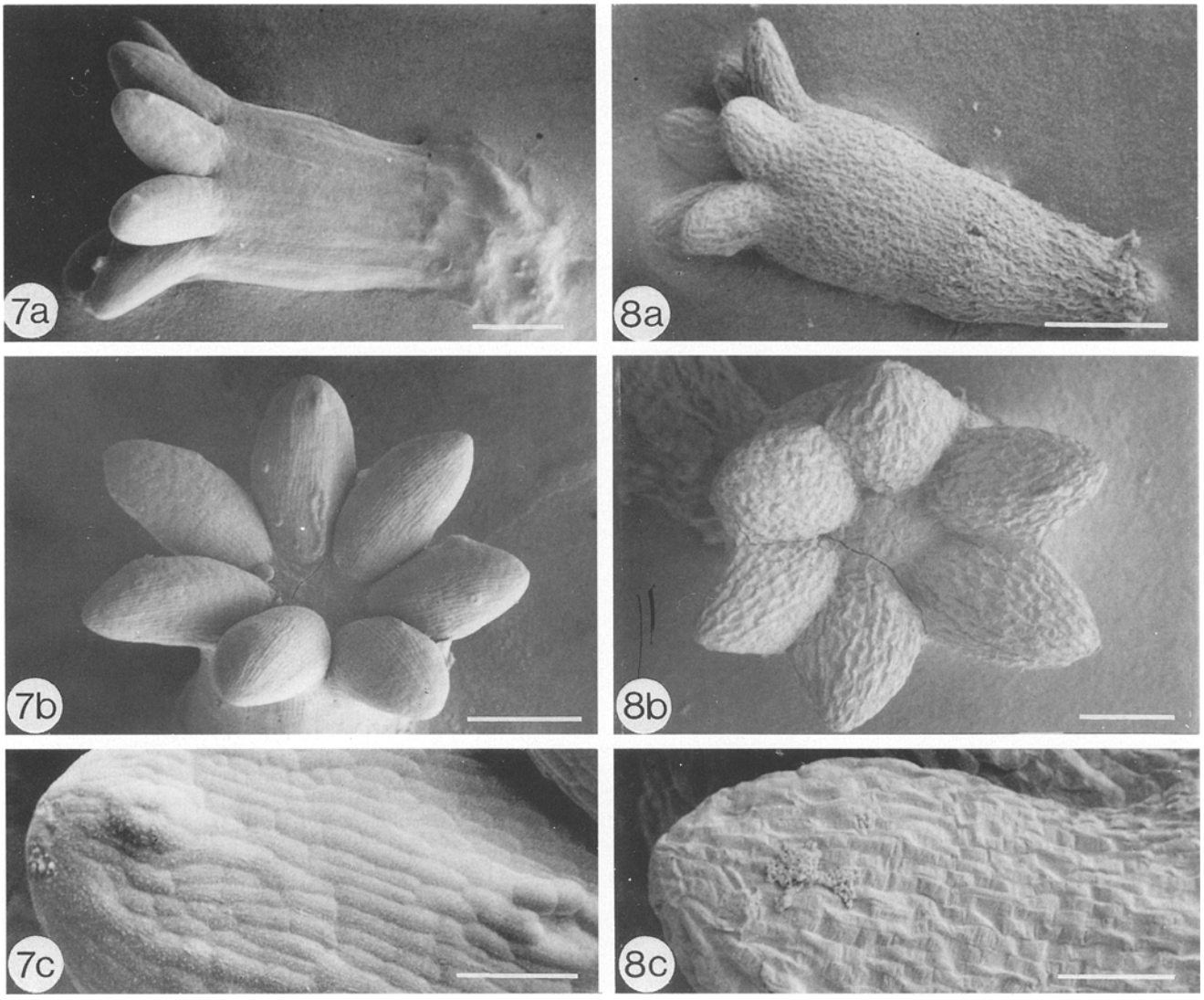

Fig. 7a. Hydrated somatic embryo examined by LTSEM (method 3), bar = 0.4 mm. **Fig. 7b**. Cotyledons and apical meristem of same embryo as in 7a. Note small crack in meristem, bar = 0.4 mm. **Fig. 7c**. Cell detail of cotyledon from same embryo as in 7a, bar = 0.1 mm. **Fig. 8a**. Desiccated somatic embryo examined by LTSEM (method 3), bar = 0.4 mm. **Fig. 8b**. Cotyledons and apical meristem of same embryo as in 8a. Note crack in meristem region, bar = 0.2 mm. **Fig. 8c**. Cell detail of cotyledon from same embryo as in 8a, bar = 0.1 mm.

(Jeffree and Read, 1991) such as the fine cracks induced by rapid freezing (Figs. 7b,8b).

Preparation Method 4: Plastic replicas. The dental impression plastic mould-cast method of Williams and Green (1988) is the least expensive of the four methods and is relatively simple. It yielded excellent replicas of both hydrated and desiccated embryos (Figs. 9,10). It is possible to make casts from a single living plant specimen at different times to follow sequential stages during maturation and growth. However, the white spruce embryos used in this study were small, delicate and in many cases quite moist making replication difficult. Embryos were thus often destroyed in the process of making replicas. The morphology of both types of somatic embryos was comparable to that provided by the other SEM methods. Some problems were encountered in completely filling the moulds thus trapping air bubbles; however the bubbles were easily recognized in the final casts (Fig. 10). Since the moulds are stable and can be reused, the process can be repeated when casts are unsatisfactory and improved casts can generally be obtained. The method is non invasive and specimens usually remain alive following mould formation.

Three stages of somatic embryo maturation

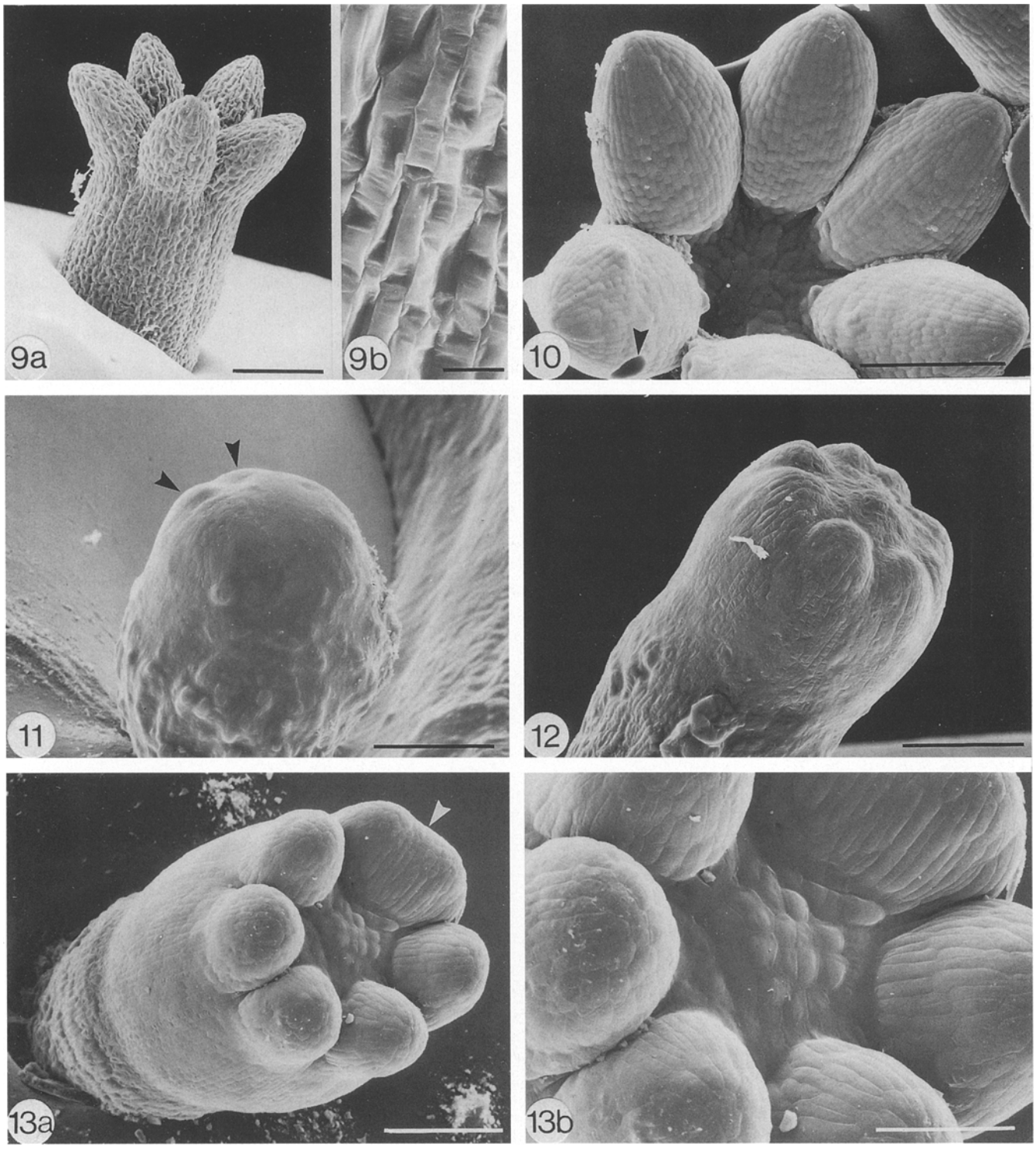

Fig. 9a. Cast of upper portion of desiccated somatic embryo (method 4), bar = 0.3 mm. **Fig. 9b**. Cell detail from same embryo as in 9a, bar = 25 μm. **Fig. 10**. Cast showing cotyledons and apical meristem of hydrated somatic embryo (method 4). Note the hole caused by an air bubble (arrow), bar = 0.3 mm. **Fig. 11**. Somatic embryo showing initiation of cotyledons (arrows) on the flattened meristematic region, bar = 0.2 mm. **Fig. 12**. Somatic embryo with domed apex and ring of cotyledons, bar = 0.2 mm. **Fig. 13a**. Somatic embryo with flared cotyledons. Note the two cotyledons which have emerged together (arrow), bar = 0.2mm. **Fig. 13b**. Enlargement from Fig. 13a showing the pattern of cells at the apex, bar = 0.1 mm.

revealed by the replica technique are illustrated in Figures 11-13. Cotyledon initiation was characterized by flattening of the meristematic region and appearance of evenly spaced small bumps surrounding the central area (Fig. 11). Subsequent growth produced a domed apex with a ring of cotyledons of similar size (Fig. 12). The cotyledons grew rapidly and quickly flared outwards (Fig. 13). Occasionally two cotyledons emerged as one large structure which later separated at the tips (Fig. 2,13).

It is clear from this study that a variety of methods are available for examining hydrated and desiccated embryos of conifers in the SEM. The simplest and least expensive method for examining dry and hydrated embryos is the plastic replica technique. It is particularly useful for morphological studies of embryo maturation. Unlike angiosperm somatic embryos, mature white spruce somatic embryos are strikingly similar in morphology to their corresponding zygotic embryos. Somatic embryos exhibited greater shrinkage than zygotic embryos, probably due to a lower density of storage lipids and lack of confinement by the megagametophyte, but still survived desiccation and germinated at high frequency.

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References

Ammirato PV (1983). In: Handbook of Plant Cell Culture, New York, MacMillan Publishing Co, V 1, pp 82-123.

Attree SM, Dunstan DI, Fowke LC (1989). Can. J. Bot. 67: 1790-1795.

Attree SM, Fowke LC (1993). Plant Cell. Tiss. Org. Cult. 35: 1-35.

Attree SM, Moore D, Sawhney VK, Fowke LC (1991). Ann. Bot. 68: 519-525.

Attree SM, Pomeroy MK, Fowke LC (1992). Planta 187: 395-404.

Attree SM, Sheffield E (1984). Micron Microsc. Acta 15: 181-186.

Brisibe EA, Nishioka D, Miyake H, Taniguchi T, Maeda E (1993). Plant Sci. 89: 85-92.

Burwale UB, Kerns HR, Widholm JM (1986). Planta 167: 473-481.

Fowke LC (1994). In: Gamborg OL, Phillips GC (Eds), Fundamental Methods of Plant Cell, Tissue and Organ Culture and Laboratory Operations, Springer Verlag, Berlin, (in press).

Hakman I, Fowke LC (1987). Plant Cell Rep. 6: 20-22.

Jeffree CE, Read ND (1991). In: Hall JL, Hawes C (Eds), Electron Microscopy of Plant Cells, Academic Press, London, pp 313-413.

Misra S, Attree SM, Leal I, Fowke LC (1993). Ann. Bot. 71: 11-22.

Robards AW, Crosby P (1979). Scanning Electron Microsc. 2: 325-344.

Vasil V, Vasil IK (1981). Ann. Bot. 47: 669-678.

Williams MH, Green PB (1988). Protoplasma 147: 77-79.

Xu N, Bewley JD (1992). Plant Cell Rep. 11: 279-284.