Protein bodies in the pine megagametophyte *in vitro* culture: ultrastructural, histochemical and electrophoretic observations

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Abstract

The megagametophytes of the European black pine (*Pinus nigra* Arn.) were cultured on modified MS medium. After 10 d, protein bodies showed well-marked degradation on freeze-etched replicas and in preparations observed by scanning electron microscopy. After 20 d of cultivation, the megagametophyte cells were completely empty. Proteins secreted into the agar medium were determined by electrophoresis and 15 different proteins, in the range of 6.5 to 71 kDa, were identified.

Additional key words: *Pinus nigra*, protein bodies degradation, proteins, SDS-PAGE.

Introduction

The seeds of the family *Pinaceae* are largely composed of a thin seed coat surrounding a massive haploid megagametophyte, within which a relatively small embryo is located. The megagametophyte is a tissue, which contains storage substances mainly in the form of lipid and proteins (De Carli *et al.* 1987). A survey of endosperm food reserves of some important crop species and also of pine megagametophytes was given by Bewley and Black (1994).

The cells forming reserve organs (for example megagametophytes and cotyledons) of conifers are very similar (Ching 1965). Lipids and proteins are distributed uniformly in the megagametophyte of Douglas fir (Owens *et al.* 1993). Besides the typical nucleus, the dormant cells of *Pinus banksiana* contain the spherosomes (fat bodies) and protein bodies, while mitochondria, dictyosomes and proplastids are not visible (Durzan *et al.* 1971). The cells of cotyledon, megagametophyte and rootlet of the dry seeds of *Pinus sylvestris* are also packed with spherosomes and protein bodies; others organelles, however, are not observable (Simola 1974).

Bonga (1974) cultured the megagametophyte tissue of *Pinus nigra* and *Pinus mugo* on Brown and Lawrence's medium in order to obtain a haploid callus. The megagametophytes of European black pine (*Pinus nigra* Arn.), Mugo pine (*Pinus mugo* Turra), Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) were pre-cultured on modified MS (Murashige and Skoog 1962) agar medium. After their removal from the medium the tester basidiomycete *Phaeolus schweinitzii* was inoculated. Very strong inhibition of mycelial growth by substances diffused from pines megagametophytes was found (Hřib *et al.* 1998). The aim of this study was to elucidate structural and ultrastructural changes in the megagametophyte cells of European black pine (*Pinus nigra* Arn.), cultured on MS agar medium, resulting from mobilization of seed reserves in protein bodies, and to carry out SDS-PAGE analysis of proteins diffused into the agar medium after 20 d of cultivation *in vitro*.

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Abbreviations: BAP - 6-benzylaminopurine; DTT - dithiothreitol; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; NAA - α-naphthaleneacetic acid.
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Materials and methods

Plants: Megagametophytes of European black pine (*Pinus nigra* Arn. ssp. *nigra*) were isolated after sterilization in 70 % (v/v) ethanol (1 min) followed by 10 - 15 min treatment in 0.1 % $HgCl_2$, washing and imbibition (24 h) of seeds at 24 ± 1 °C. The megagametophytes were cultured on modified MS (Murashige and Skoog 1962) agar medium containing 5 mg dm^{-3} NAA and 0.1 mg.dm^{-3} BAP (Hřib and Rypáček 1981). The cultures were maintained at 24 ± 1 °C in the darkness.

Histochemical study: The megagametophytes were cut in longitudinal planes to get slices of tissue 1 - 2 mm thick. These were fixed in 3 % (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.0) for 2 h at 23 °C, dehydrated using a 50 to 100 % (v/v) ethanol series, transferred to ethanol: toluene (3:1, 2:2, 1:3), pure toluene and finally embedded in paraffin. Sections 8 to 10 μm thick were made. To aid photography, the background was enhanced by staining mounted sections first with 0.05 % (m/v) aqueous toluidine blue. This was followed by protein-staining, using 1 % (m/v) $HgCl_2$ and 0.05 % (m/v) bromophenol blue in 2 % (v/v) aqueous acetic acid (Pearse 1968).

Scanning electron microscopy: Samples of megagametophytes were cut and fixed immediately after collection and after 10 d and 20 d of cultivation, using 3.5 % glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, for 24 h and 2 % OsO_4 in the same buffer for 10 h. The fixed tissues were rinsed 3 times in phosphate buffer and dehydrated in a graded ethanol series (30 - 100 %) and then subjected to critical point drying with CO_2 as a transition fluid. The surface of cross and longitudinal sections was coated with gold and examined and photographed in a *Tesla BS 300* (Brno, Czech Republic) scanning electron microscope (SEM).

Freeze-fracturing: Samples of megagametophytes were fixed immediately after collection and after 10 d and 20 d of cultivation with 3.5 % glutaraldehyde in 0.05 M phosphate buffer, pH 7.4, for 24 h. The fixed tissues were rinsed 3 times in phosphate buffer and transferred to an ascending series of glycerol (5, 10 and 20 %). Subsequently, the samples were studied by freeze-fracturing, using a *BA 360 M Balzers* (Lichtenstein) apparatus. Platinum-carbon replicas were examined and photographed in a *Tesla BS 500* electron microscope.

Protein determination: The protein concentration was measured by the Coomassie blue (Coomassie Brilliant Blue G 250, *Serva*, Heidelberg, Germany) method of Bradford (1976). A standard curve was made with bovine serum albumin (*Sigma*, St. Louis, USA).

Gel electrophoresis: Agar samples with substances exuded (diffused) from about 30 megagametophytes cultured on modified MS medium with 5 mg.dm^{-3} NAA and 0.1 mg.dm^{-3} BAP in Petri dishes for 20 d were used for protein analysis by SDS-PAGE. The agar medium with extracellular substances was homogenized with a half volume of the 3 × Reducing Sample Buffer (*BioLabs*, New England, USA). SDS-PAGE and staining with Coomassie Blue were performed according to the method of Laemmli (1970) with a slight modification (Smith and Sasse 1987). Proteins were reduced by DTT. Stacking and separation gels were with 4 % and 13 % (m/v) acrylamide, respectively. Molecular masses of protein standards of *BioLabs* were: myosin 212 kDa, MBP-β-galactosidase 158.2 kDa, β-galactosidase 116.3 kDa, phosphorylase *b* 97.2 kDa, serum albumin 66.4 kDa, glutamic dehydrogenase 55.6 kDa, maltose-binding protein 42.7 kDa, lactate dehydrogenase M 36.5 kDa, triosephosphate isomerase 26.6 kDa, trypsin inhibitor (soybean) 20.0 - 20.2 kDa, lysozyme 14.3 kDa, aprotinin 6.5 kDa, insulin 2.3 - 3.4 kDa. The gels were analysed by means of the *Image Master DTS* from *Pharmacia LKB* (Uppsala, Sweden).

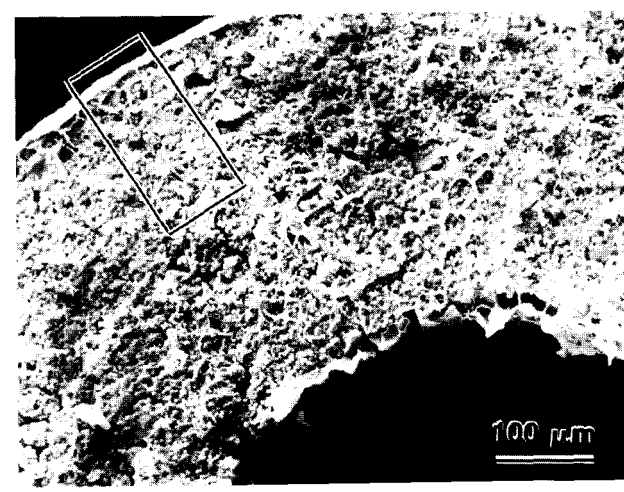

Fig. 1. SEM micrograph of pine megagametophyte. The following Fig. 2 is approximately from marked region.

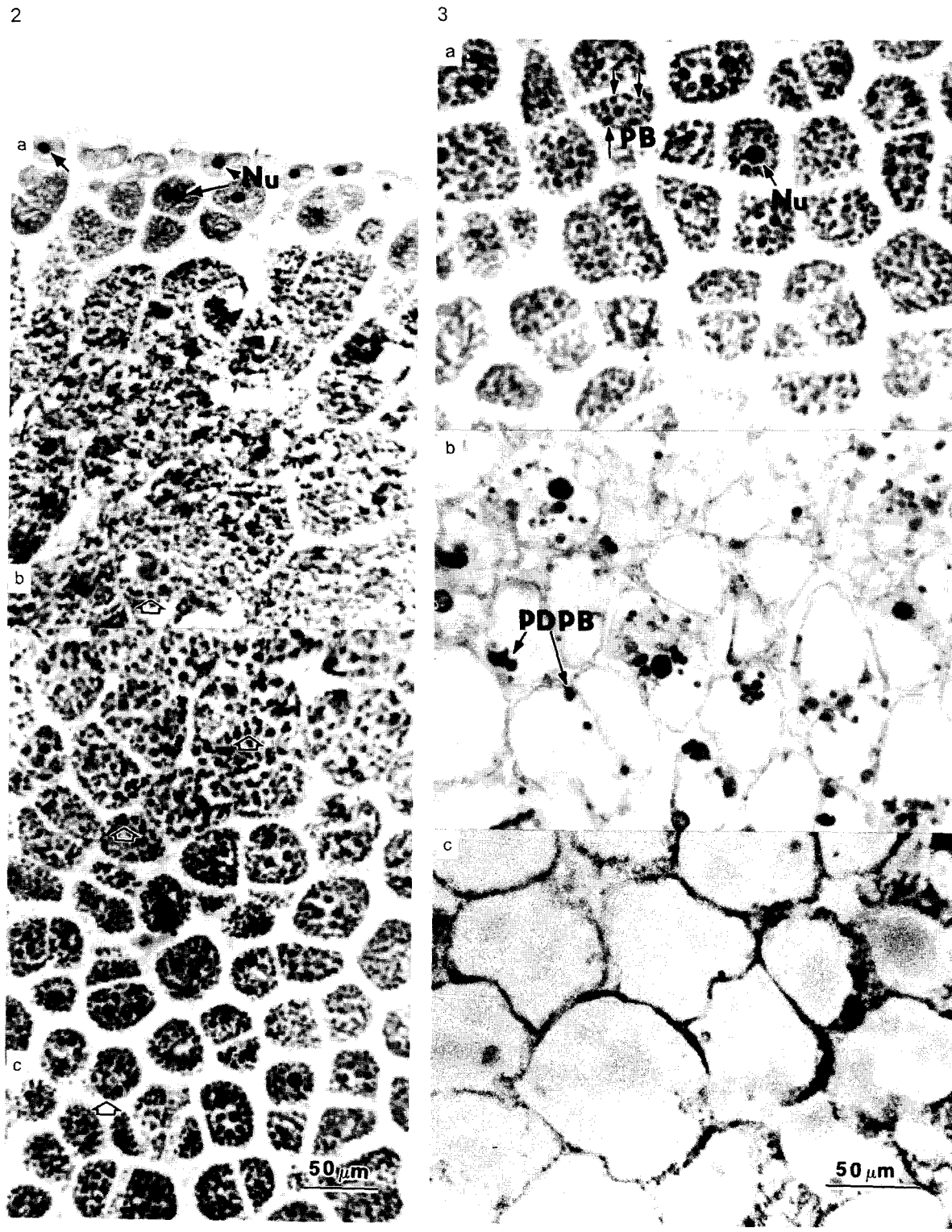

Fig. 2. Light microscopy of pine megagametophyte (*a* - surface part of the megagametophyte with flat cells, *b* - parenchymatic cells of megagametophyte, *c* - central part of megagametophyte with sclerenchymatic cells; Nu - nucleus; *arrows* point to protein bodies).

Fig. 3. Light microscopy of pine megagametophyte cells cultured *in vitro* (*a* - 0 d, *b* - after 10 d, *c* - after 20 d; Nu - nucleus, PB - protein bodies, PDPB - partly degraded protein bodies).

Results and discussion

The megagametophyte of European black pine (*Pinus nigra* Arn., ssp. *nigra*), formerly *P. nigra* var. *austriaca* (Hoess) Loud, consisted of three parts of different types of tissues (Figs. 1, 2).

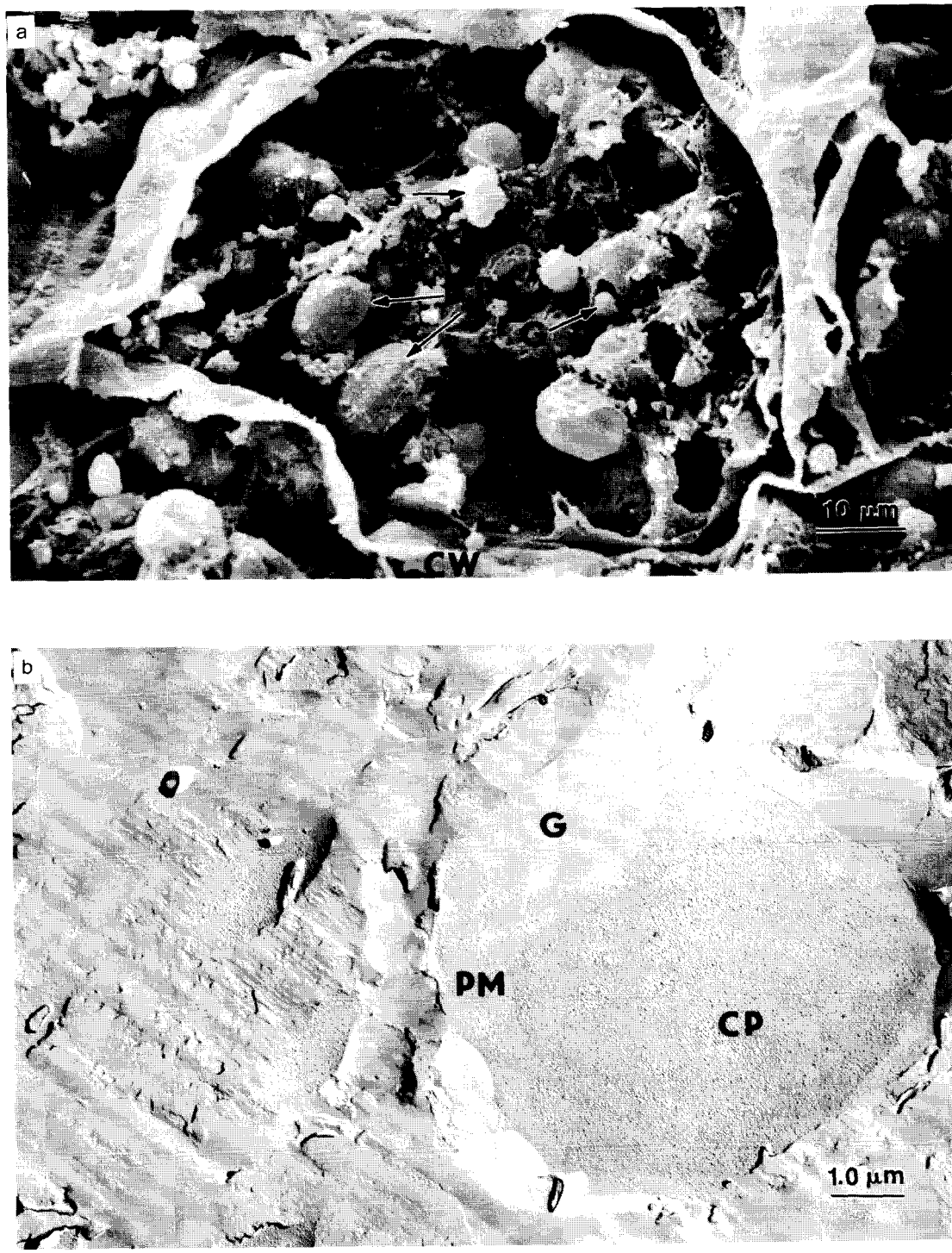

Fig. 4. Ultrastructure of pine megagametophyte cells immediately after extirpation (*a* - SEM micrograph of megagametophyte cells; *b* - freeze-fracturing micrograph of megagametophyte cell; PB - protein bodies, G - globoid, CP - crystalloid protein complex, PM - proteinaceous matrix, S - spherosomes).

The surface of the megagametophyte was covered by flat, tile-like cells with distinct nuclei. The part below the surface consisted of large parenchymatic cells closely attached to each other and rich in protein bodies. The central part of the megagametophyte contained sclerenchymatic cells with a characteristic structure which were not too closely arranged and were also rich in protein bodies (Fig. 2).

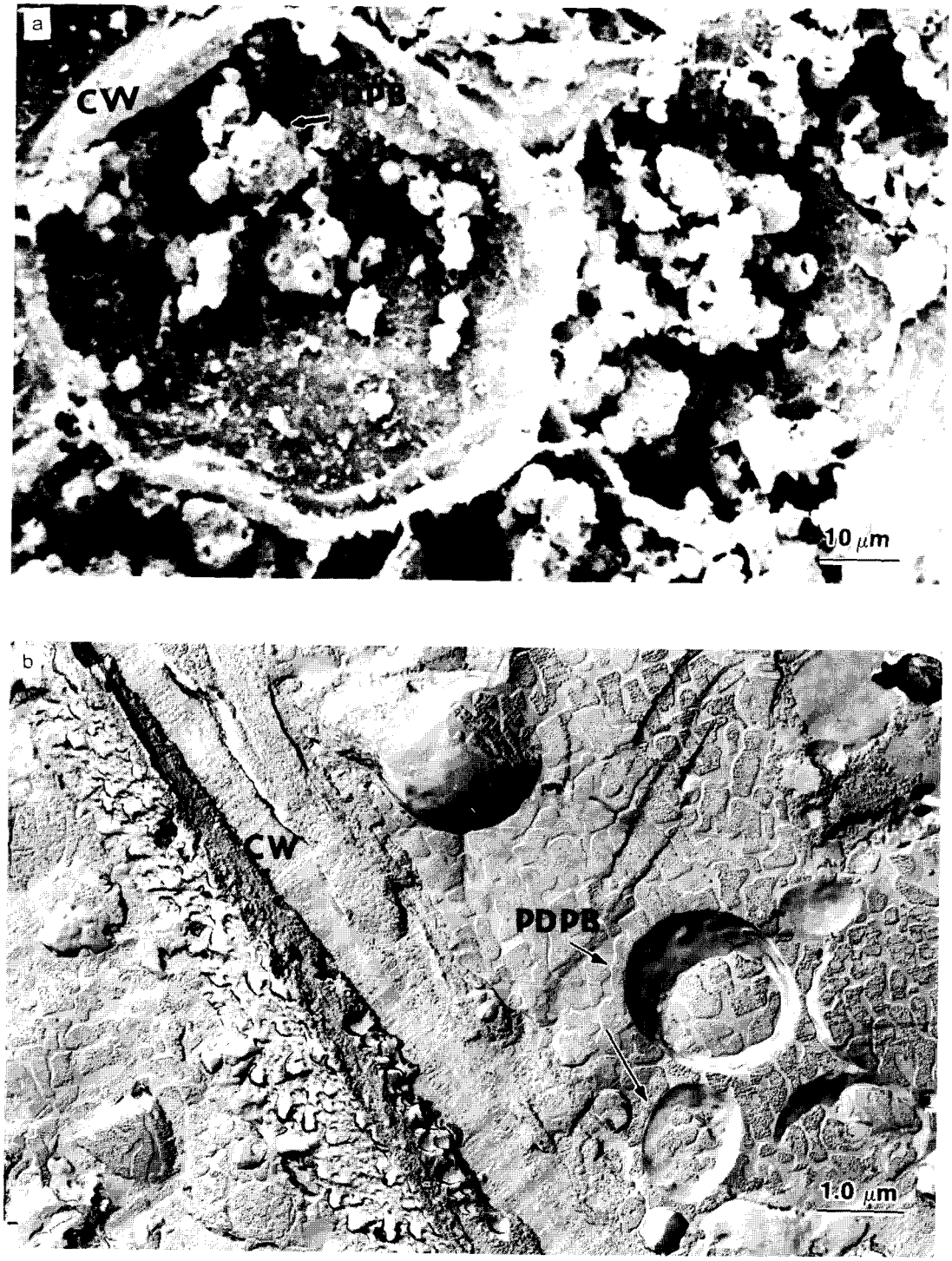

Fig. 5. Ultrastructure of pine megagametophyte cells after 10 d of *in vitro* cultivation (*a* - SEM micrograph of megagametophyte cells, *b* - freeze-fracturing micrograph of megagametophyte cell; PDPB - partly degraded protein bodies, CW - cell wall).

The protein bodies usually contained a globoid and a crystalloid protein (Fig. 4*b*). After 10 d of megagametophyte culture *in vitro*, the protein bodies showed marked degradation (Figs. 3*b*, 5*a*,*b*) and cell organelles were found only on the periphery of the cell near the cell wall (Fig. 5*b*). After 20 d of cultivation, the cells were completely empty without any protein bodies (Figs. 3*c*, 6*a*,*b*).

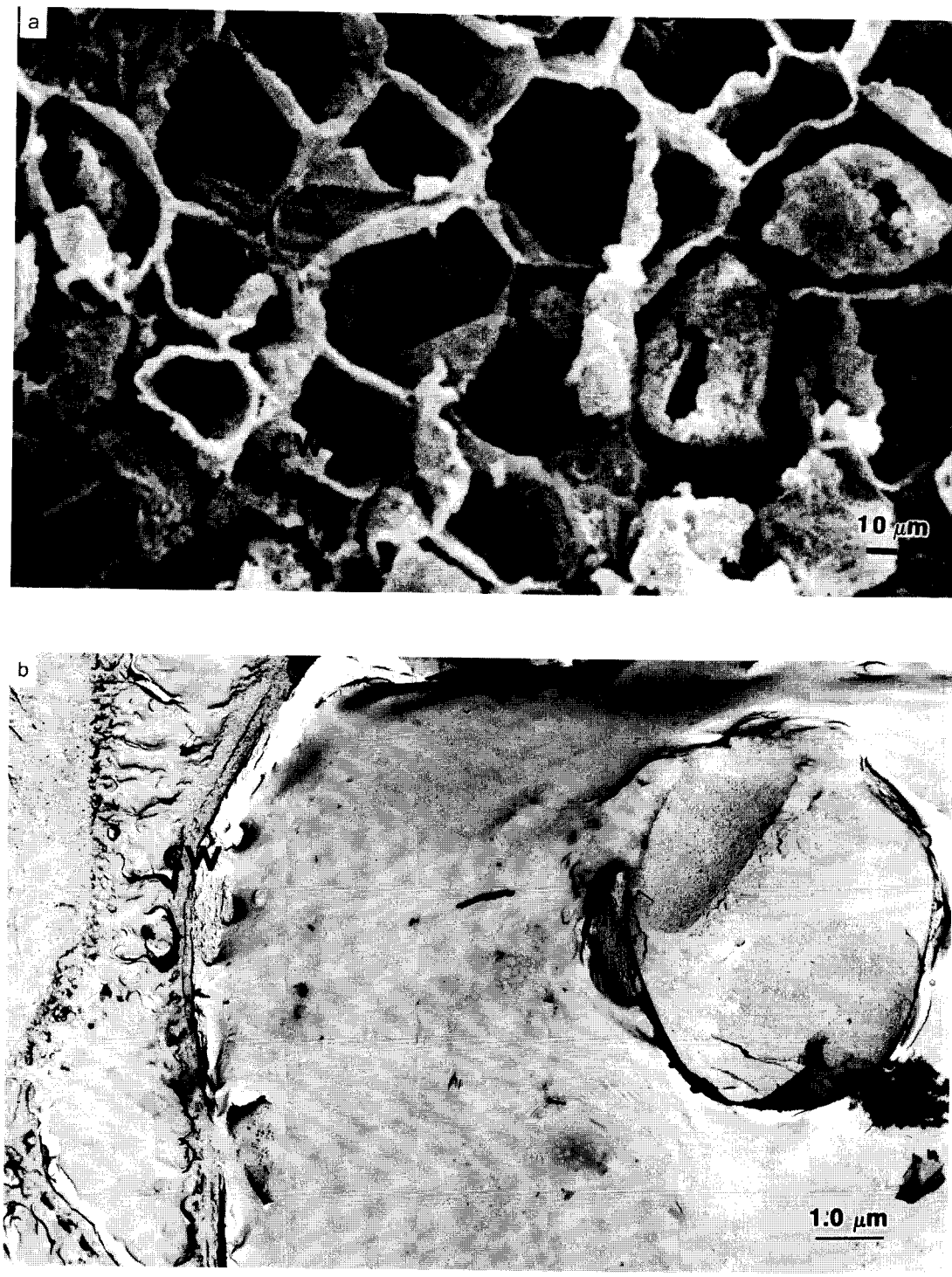

Fig. 6. Ultrastructure of pine megagametophyte cells after 20 d of *in vitro* cultivation (*a* - SEM micrograph of megagametophyte cells, *b* - freeze-fracturing micrograph of megagametophyte cell; CW - cell wall).

After 20 d of cultivation, proteins (polypeptides) exuded into agar medium were prepared for analysis and their electrophoretic behaviour was determined by SDS-PAGE. The Coomassie blue stained profile, obtained after separating these proteins, had a set of 15 proteins with molecular masses of 6.5, 8, 12.5, 22, 25, 26.5, 28.5, 36.5, 38, 38.5, 51.5, 52.5, 60, 64 and 71 kDa (Fig. 7).

Gifford (1988) showed that major storage proteins of mature *Pinus monticola* Dougl. seed megagametophytes originated from the crystalloid and were completely soluble in buffer solutions only if sodium dodecyl sulfate was present. These insoluble proteins constituted 50 % of the storage reserves in seeds. Crystalloid proteins were also found in embryonic axis and provided a major protein reserve in mature seeds in all other *Pinus* species examined. These proteins, in reduced forms migrate on polyacrylamide gels as two distinct groups of proteins, one in the molecular mass range of 21.5 - 22.5 and the other in the 31 - 34.5 kDa range.

In previous study, *P. nigra* protein bodies were also isolated from mature seeds. Extracted soluble and insoluble (crystalloid) protein body proteins were investigated by SDS-PAGE in both the presence and absence of 2-mercaptoethanol (Hajduch *et al.* 2000).

Under *in vitro* conditions, Bonga (1974) has observed that pine megagametophyte culture regenerate after 5 to 7 weeks and a callus is formed. Under our experimental conditions callogenesis was observed on the surface of megagametophytes when these megagametophytes were cultured longer than one month.

During cultivation of the megagametophyte, the cells underwent expressive changes in storage substances. During 20 d of megagametophyte culture the total protein body content was mobilized and its hydrolysates diffused into agar medium.

Many plant species constitutively accumulate toxic, low molecular compounds (*e.g.* antibiotics, saponins, alkaloids, glycosides, cyanide-generating compounds) and various types of toxic proteins in their tissues as a means of passive defence. Some of these proteins are very potent toxins with a broad spectrum, they include ribosome-inactivating proteins (RIP) and lectin-like toxins (such as ricin and abrin). Other exhibit a selective toxicity against a particular group of organisms (*e.g.* antifungal proteins, lytic enzymes, insecticidal proteins, alfa-amylase inhibitor, lectins) (Peumans and Van Damme 1995). Tsuda (1979) described rice lectin polypeptides with values of 8, 10 and 18 kDa. Peumans *et al.* (1984) isolated an unusual lectin from stinging nettle (*Urtica dioica* L.) rhizomes. This is a small (8.5 kDa) monomeric protein with high content of glycine, cysteine and tryptophan. The *U. dioica* agglutinin (UDA) is specifically inhibited by N-acetylglucosamine oligomers. Nahálková *et al.* (1996) found that crude protein bodies extracts from *P. nigra* seeds have the lectin-like activity specific for chitin and chitooligosaccharides and also positive activity for the chitinase. Nahálková *et al.* (1999), also described that crude protein fraction isolated from protein bodies possessed an ability to agglutinate fungal spores. Inhibition of the fungal spores agglutination confirmed the presence of lectin-like proteins specific to basic fungal cell wall components.

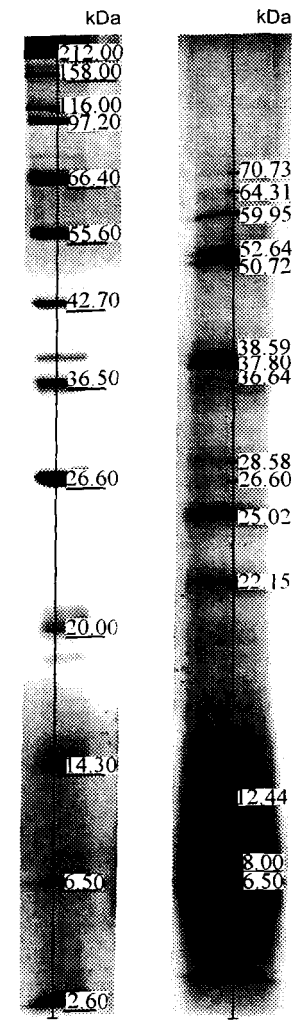

Fig. 7. Coomassie blue stained SDS-PAGE profile of proteins from the agar medium after 20 d of pine megagametophyte culture. 100 μg of protein was applied to line.

It is probable that 8, 9, 13 kDa and 18 kDa proteins play a role in defense of pine seed tissue (Vooková *et al.* 1999). In this study, after 20 d of megagametophyte culture *in vitro*, 8 and 12.5 kDa polypeptides were repeatedly found in agar medium. Experiments concerning isolation and characterization of lectin-like proteins from pine seeds will be carried out in the future.

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