BIOLOGIA PLANTARUM 45 (4): 539-544, 2002

Some properties of proteolytic enzymes and storage proteins in recalcitrant and orthodox seeds of *Araucaria*

L. GALLESCHI, A. CAPOCCHI, S. GHIRINGHELLI and F. SAVIOZZI

Department of Botanical Sciences, University of Pisa, Via L. Ghini 5, I-56126 Pisa, Italy

Abstract

Araucaria bidwillii Hook. and *Araucaria cunninghamii* Don D. are two species of conifers whose seeds belong to different physiological categories: *A. bidwillii* seeds are recalcitrant, while *A. cunninghamii* seeds are orthodox. The extraction of enzymes and storage proteins was carried out from *A. bidwillii* and *A. cunninghamii* megagametophytes. The endopeptidase activities of both species were assayed with azocasein and with haemoglobin; the exopeptidase activities were detected by various N-carbobenzyloxy-dipeptides and L-leucine *p*-nitroanilide. The use of appropriate proteinase inhibitors, *i.e.* pepstatin A, ethylenediaminetetraacetic acid and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, showed the presence of aspartic and metallo proteinases and the absence of the cysteine ones both in *A. bidwillii* and in *A. cunninghamii* ungerminated seeds. Since the results do not show differences between the types of enzymes in the ungerminated araucarian seeds and those present in some ungerminated angiosperm seeds (barley, wheat, maize, rice, buckwheat), we conclude that their physiological role is similar. The electrophoretical analyses of soluble and insoluble storage proteins of *A. cunninghamii* showed patterns similar to those found in other gymnosperms, while the storage protein patterns of *A. bidwillii* seeds were rather atypical.

Additional key words: *Araucaria bidwillii* Hook., *Araucaria cunninghamii* Don D., globulins, megagametophyte, protease inhibitors, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

Araucarias are coniferous trees which originate from regions with a tropical or sub-tropical climate. *Araucaria cunninghamii* Don D. (hoop pine) occurs in the forests of coastal regions of eastern Australia and in the elevated areas of Papua New Guinea. *Araucaria bidwillii* Hook. (bunya pine) occurs sparsely in south-eastern Queensland, generally in association with *A. cunninghamii*. The bunya pine seed is large, heavy, starchy and at maturity its moisture content is around 48 %; the hoop pine seed is small, light, oleaginous and its moisture content is around 7 %. So the two species belong to different physiological categories as regards the viability of seeds during storage (Roberts 1973): recalcitrant seeds (*A. bidwillii,* Del Zoppo *et al.* 1998b) and orthodox seeds (*A. cunninghamii*).

Knowledge about seed storage protein mobilisation in gymnosperms is very scanty. A partial characterisation of several proteolytic activities in dry and germinating seeds has been carried out with *Pinus sylvestris* L. (Salmia 1981), with *Pinus contorta* Dougl. (Gifford *et al.* 1989) and with *Picea glauca* (Moench) Voss (Gifford and Tolley 1989). The storage proteins, typical of these seeds, have been characterized in several species from the *Pinaceae, Cupressaceae* and *Taxaceae* families (Allona *et al.* 1994).

Received 18 June 2001, *accepted* 29 November 2001.

Abbreviations: CBZ-Ala-Arg - N-carbobenzyloxy-L-alanyl-L-arginine; CBZ-Phe-Ala - N-carbobenzyloxy-L-phenylalanyl-L-alanine; CBZ-Phe-Phe - N-carbobenzyloxy-L-phenylalanyl-L-phenylalanine; CBZ-Phe-Pro - N-carbobenzyloxy-L-phenylalanyl-L-proline; CBZ-Pro-Ala - N-carbobenzyloxy-L-prolyl-L-alanine; *p*-CMPS - *p*-chloromercuriphenylsulfonic acid; E-64 - trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; EDTA - ethylenediaminetetraacetic acid; LPA - L-leucine *p*-nitroanilide; 2-ME - 2-mercapto-ethanol; *o*-phen - *o*-phenanthroline; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF - phenylmethanesulfonyl fluoride; Tris - tris(hydroxymethyl)aminomethane.

Acknowledgments: We would like to thank Dr. S. D'Alessandro for her support during the study and the colleagues at the Botanical Gardens of Pisa (Italy) and the Department of Primary Industries (Beerwah, Queensland, Australia) for supplying the seeds.

Fax: (+39) 50 551345, e-mail: lgalles@dial-up.unipi.it

The aim of this paper is an analysis of proteolytic enzyme activities and storage proteins that are present in mature seeds of these two araucarian species. In particular, we have tried to identify whether their morphological and physiological differences are correlated with a different (pattern) types of proteolytic enzymes or with different storage proteins of ungerminated seeds.

Materials and methods

A. bidwillii Hook. seeds were collected at the end of August 1998 from plants growing in the Botanical Gardens of the University of Pisa; *A. cunninghamii* Don D. seeds were supplied by the Department of Primary Industries (Beerwah, Queensland, Australia). The megagametophytes from seeds of *A. bidwillii* and *A. cunninghamii* were manually isolated, lyophilized and dry homogenized with a mortar and pestle. The flour, thus obtained, was utilized as enzymatic source and for the extraction of storage proteins.

Flour of *A. bidwillii* and *A. cunninghamii* megagametophytes (6 g) was extracted according to Del Zoppo *et al.* (1998b). Carboxypeptidase activity from extracts was assayed with N-carbobenzyloxy-Phe-Ala, CBZ-Pro-Ala, CBZ-Phe-Phe, CBZ-Ala-Arg and CBZ-Phe-Pro. Aminopeptidase activity was assayed with L-leucine *p*-nitroanilide (Del Zoppo *et al.* 1998a). The same exopeptidase assays were performed in the presence of phenylmethanesulfonyl fluoride (1 mM final conc.) for the carboxypeptidases, and *p*-chloromercuriphenyl-sulfonic acid (1 mM final conc.) and *o*-phenanthroline (3 mM final conc.) for the aminopeptidases. Haemoglobinase activity was determined according to Galleschi *et al.* (1989). We assessed the effects of several inhibitors on this enzymatic activity: trans-epoxysuccinyl-L-leucyl-amido-(4-guanidino) butane (20 μM final conc.), pepstatin A (1.43 μM final conc.), ethylenediamine-tetraacetic acid (1 mM final conc.), and PMSF (1 mM final conc.). The exopeptidase and endopeptidase activities were expressed as pkatals g^{-1}(flour) (Florkin and Stotz 1973). Azocaseinase activity was assayed with azocasein at pH 5.4 and pH 7.0. The proteinase activity was determined according to Del Zoppo *et al.* (1999). The two azocaseinase activities were also assayed in the presence of the same inhibitors previously used. The enzymatic activity was expressed as units of protease activity g^{-1}(flour) (Sarath *et al.* 1989).

The storage proteins of *A. bidwillii* and *A. cunninghamii* were extracted following Gifford *et al.* (1982) and their protein content was determined according to Bensadoun and Weinstein (1976). 50 mm^3 (180 μg of proteins) of *A. bidwillii* and *A. cunninghamii* soluble proteins were mixed with an equal volume of sample buffer (Laemmli 1970), containing or not 2-mercaptoethanol and boiled for 3 min. *A. bidwillii* and *A. cunninghamii* insoluble proteins were directly used for sodium dodecyl sulfate polyacrylamide gel electrophoresis after the addition of 0.05 % bromophenol blue (m/v). SDS-PAGE of soluble and insoluble proteins was performed on minislabs (*Dual Vertical Minigel* unit, *C.B.S.*), according to Laemmli (1970). An electrophoretic run was carried out for 1 h and 30 min under constant current conditions. Approximately 15 μg of soluble and insoluble proteins were loaded per lane. The gels were calibrated with 12 mm^3 of high molecular mass and low molecular mass protein standards (*BDH*, Poole, England). Following electrophoresis the gels were stained overnight in 0.025 M (m/v) Coomassie Brilliant Blue R 250 (*Sigma*, St. Louis, USA) according to Koenig *et al.* (1970) and destained in 7 % acetic acid. The gels were scanned and processed by a digital image analysis program (*SigmaGel*, *Jandel Corporation*, San Rafael, USA).

Results and discussion

One of the most notable carboxypeptidase activities present in *A. cunninghamii* and *A. bidwillii* is the one affecting CBZ-Phe-Ala (Fig. 1) as found in wheat seeds (Mikola 1986) and in several other species. The other high carboxypeptidase activity against CBZ-Pro-Ala, probably correlated to wheat prolylcarboxypeptidases (Mikola 1986), does not seem to have the same role, since proline is not a frequent amino acid in gymnosperm storage proteins (King and Gifford 1997). These storage proteins contain, in fact, a large amount of arginine and

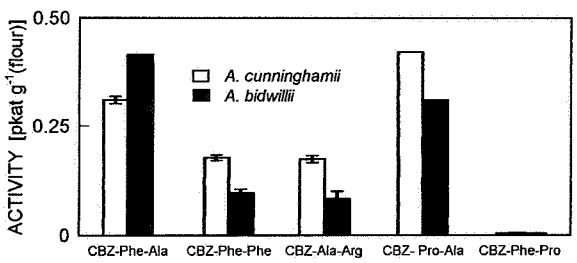

Fig. 1. Carboxypeptidase activity in *A. cunninghamii* and *A. bidwillii* ungerminated seeds.

glutamine, so the enzymatic activity hydrolyzing CBZ-Ala-Arg may be particularly important during germination, in order to degrade the hydrolysis products containing arginine. The activity hydrolyzing CBZ-Phe-Phe is markedly lower than the others, as in dry and germinating seeds of *P. sylvestris* (Salmia and Mikola 1976). PMSF almost completely inhibits CBZ-Phe-Ala hydrolysis in both species and CBZ-Phe-Phe hydrolysis in *A. bidwillii* (Table 1). These data confirm the serine nature of araucarian carboxypeptidases (Ryan and Walker-Simmons 1981). On the contrary, CBZ-Ala-Arg hydrolysis is not very sensitive to PMSF and CBZ-Pro-Ala hydrolysis is nearly completely insensitive (Table 1). This result is quite unusual for plant carboxypeptidases, so further studies are needed to verify whether these activities could be assigned to metallo-carboxypeptidases isolated by Doi *et al.* (1980) in rice shoots. The second type of exopeptidase is more abundant than the first, above all in *A. cunninghamii* seeds, where aminopeptidase activity is 70-80 times higher than

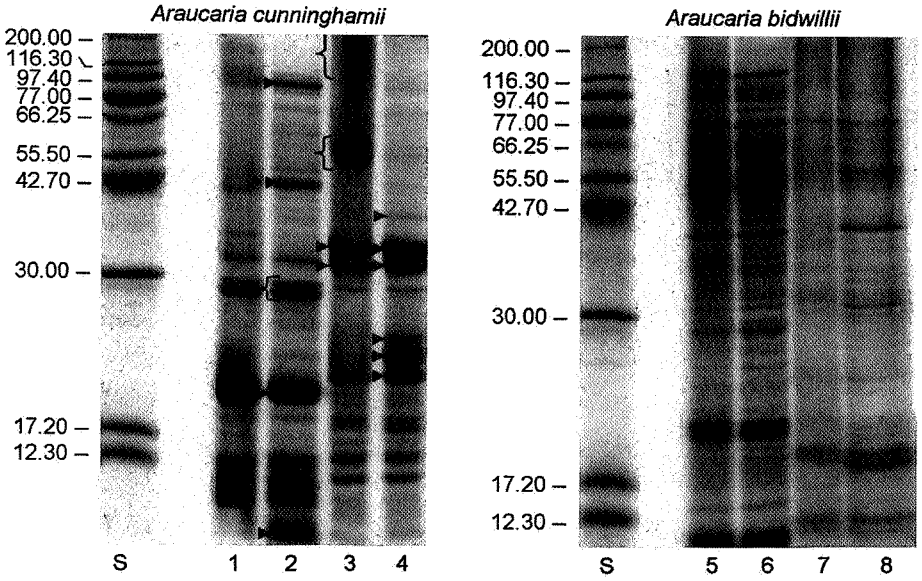

Fig. 2. SDS-PAGE of soluble (*lanes* 1, 2 and 5, 6) and insoluble (*lanes* 3, 4 and 7, 8) proteins, in presence (*lanes* 2, 4 and 6, 8) or in absence (*lanes* 1, 3 and 5, 7) of 2-mercaptoethanol extracted from *A. cunninghamii* and *A. bidwillii* ungerminated seeds. Molecular mass markers (S): myosin (200 kDa), b-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), ovotransferrin (76 - 78 kDa), albumin (66.25 kDa), glutamate dehydrogenase (55.5 kDa), ovalbumin (42.7), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa), cytochrome *c* (12.3 kDa).

Table 1. Inhibition of the carboxypeptidase activity of *A. bidwillii* and *A. cunninghamii* ungerminated seeds by PMSF.

Substrates	*A. bidwillii* control [pkat g^{-1} (flour)]	inhibition [%]	*A. cunninghamii* control [pkat g^{-1}(flour)]	inhibition [%]
CBZ-Phe-Ala	0.410 ± 0.0045	89.2	0.31 ± 0.0190	78.9
CBZ-Phe-Phe	0.098 ± 0	100	0.18 ± 0.0082	63.6
CBZ-Ala-Arg	0.084 ± 0.0085	57.8	0.17 ± 0.0065	18.3
CBZ-Pro-Ala	0.310 ± 0.016	2.59	0.42 ± 0.0083	2.6

carboxypeptidase activity with CBZ-Phe-Ala (Tables 1, 2). This result is analogous to that observed in *P. sylvestris* (Salmia and Mikola 1975) and in *P. contorta* (Gifford *et al.* 1989). *p*-CMPS inhibition (Table 2) confirms the presence of SH-groups in the active site of these enzymes; *o*-phenanthroline inhibits only *A. cunninghamii* extract up to 85 % (Table 2). Probably in this case the enzymes need some bivalent cations to stabilize or even to increase their activity as found in rice (Ryan and Walker-Simmons 1981). However, there is some uncertainty as to the role of these enzymes during seed germination; in fact, the highest aminopeptidase activities were present in the endosperms throughout the period of storage protein mobilization, while carboxypeptidases were observed in the endosperms of germinating pine seeds at the stage when the storage proteins were completely depleted. It

seems, therefore, that only aminopeptidases can be involved in storage protein degradation, while carboxypeptidases could have a role in the proteolytic reactions associated with the senescence of the endosperm of germinating gymnosperm seeds (Salmia and Mikola 1976, Gifford *et al.* 1989). A work in progress in our laboratory about the role of proteolytic enzymes during germination of *A. bidwillii* seeds, seems to confirm this hypothesis.

Table 2. Inhibition [%] of the aminopeptidase activity of *A. bidwillii* [control value: 2.72 ± 0.44 pkat g^{-1}(flour)] and *A. cunninghamii* [control value: 31.6 ± 1.45 pkat g^{-1}(flour)] ungerminated seeds by *p*-CMPS and *o*-phenanthroline.

Inhibitors	*A. bidwillii*	*A. cunninghamii*
p-CMPS	97	94
o-phenanthroline	19	85

Table 3. Inhibition [%] of endopeptidase activities of *A. bidwillii* ungerminated seeds [haemoglobinase control value: 0.144 ± 0.018 pkat·g^{-1}(flour); azocaseinase pH 5.4 control value: 5.26 ± 0.7 U g^{-1}(flour) × 10^{-3}; azocaseinase pH 7.0 control value: 3.52 ± 0.42 U g^{-1}(flour) × 10^{-3}] and *A. cunninghamii* ungerminated seeds [haemoglobinase control value: 0.363 ± 0.014 pkat g^{-1}(flour); azocaseinase pH 5.4 control value: 16.2 ± 0.52 U g^{-1}(flour) × 10^{-3}; azocaseinase pH 7.0 control value: 9.65 ± 0.57 U g^{-1}(flour) × 10^{-3}] by several inhibitors.

Inhibitors	*A. bidwilli* haemoglobin pH 3.7	azocasein pH 5.4	azocasein pH 7.0	*A. cunninghamii* haemoglobin pH 3.7	azocasein pH 5.4	azocasein pH 7.0
E-64	11	12	0	0	0	0
Pepstatin A	41	57	18	48	41	10
EDTA	0	4	45	0	5	57.5
PMSF	30	31	21	54	12	26.5

Enzymes with endopeptidase activity, present in ungerminated seeds of the two araucarian species, hydrolyse the same substrates (haemoglobin and azocasein) and show the same pH optimum for the activity as analogous enzymes from *P. sylvestris* (Salmia 1981). The use of some protease inhibitors allowed us to characterise the kinds of proteolytic enzymes involved (Table 3). Enzymes which hydrolyse haemoglobin at pH 3.7 and azocasein at pH 5.4 are sensitive to pepstatin A and PMSF in both *A. bidwillii* and in *A. cunninghamii* (Table 3). The protease activity extracted from *P. sylvestris* is deeply inhibited (90 %) by pepstatin A suggesting the presence of high levels of aspartic proteinases (Salmia 1981). Similar enzymes have been found in dry seeds of monocotyledons and dicotyledons, where they might perform the role of starting proteinases (Galleschi 1998), although in developing barley seeds they are not secreted in starchy endosperm during germination (Tormakangas *et al.* 1994). Aspartic proteinases may, however, participate in the degradation of storage proteins modified by the action of cysteine proteinases (Capocchi *et al.* 2000). Both extracts at pH 3.7 are inhibited by PMSF, while at pH 5.4 only *A. bidwillii* extract is sensitive to this inhibitor (Table 3). This treatment confirms the presence of serine carboxypeptidases. EDTA inhibition at pH 7.0 shows the presence of high metallo-proteinase activity (Table 3), which could be involved in the initial stages of storage protein degradation. Since E-64 did not influence enzymatic activity at all (Table 3), cysteine proteinases are absent in araucarian ungerminated seeds. A similar result has been found in cereal seeds (Galleschi *et al.* 1989, Capocchi *et al.* 2000), where these enzymes reach their maximal activity a few days after germination when maximal storage protein hydrolysis occurs (Galleschi 1998).

Since the two species of ungerminated araucarian seeds differ only in the levels of proteolytic activity, we suggest that a similar enzymatic machinery is responsible for the degradation of storage proteins in our seeds.

Insoluble globulins usually represent the main protein reserve in gymnosperms (Allona *et al.* 1994). However, a quantitative analysis of soluble and insoluble proteins of *A. bidwillii* and *A. cunninghamii* seeds shows that in *A. cunninghamii* the soluble ones are markedly prevalent: 135.26 *vs.* 39.3 mg(insoluble proteins) g^{-1}(flour). In *A. bidwillii* both protein types are equally represented: 41.33 and 40.16 mg(soluble proteins) g^{-1}(flour). The notable presence of an easily available energetic source (soluble proteins) could be particularly important for species with recalcitrant seeds such as *A. bidwillii*. SDS-PAGE analysis of soluble proteins of *A. cunninghamii* shows that the electrophoretic behaviour of some major components (94, 42, 28 - 30, 20 kDa) is not altered in the presence of 2-ME (Fig. 2; *lanes* 1 and 2), although some bands (94, 42 kDa) become more

intense. A similar pattern has been observed in *Pinus monticola* Dougl. (Gifford 1988) and *P. pinaster* (Allona *et al.* 1992). Other protein components with molecular mass lower than 12.3 kDa are sensitive to 2-ME; these could be arginine-rich proteins corresponding to 2S globulins of some angiosperm seeds (Allona *et al.* 1994). *A. cunninghamii* insoluble proteins extracted in the absence of 2-ME show a number of main components with molecular masses 117 - 179, 51 - 59, 34, 31 kDa (Fig. 2, *lane* 3). Under reducing conditions (Fig. 2, *lane* 4) the components with molecular mass higher than 50 kDa split into polypeptides of lower molecular mass whose bands appear with major intensity (34, 31 and 22 kDa) or as new bands (38, 25 and 23 kDa). A similar behaviour has been shown in *P. monticola*, where high molecular mass insoluble proteins (higher than 100 kDa) are not detectable when the extraction is performed in the presence of 2-ME and a major set of proteins with masses in the range 51 - 55 kDa split into two sets of proteins with molecular masses ranging between 31.5 and 35 and between 21 and 22.5 (Gifford 1988). Electrophoretic analysis of *A. bidwillii* soluble and insoluble proteins showed a very different pattern from that observed in *A. cunninghamii*: the soluble globulins remains unchanged following the 2-ME treatment (Fig. 2, *lanes* 5, 6), while the insoluble ones don't show bands with molecular mass around 50 - 60 kDa which split in the presence of the reducing agent into polypeptides with lower molecular mass (Fig. 2, *lanes* 7, 8). The occurrence in coniferous seeds of storage proteins homologous to angiosperm storage globulins (Haider and El-Shanshoury 2000, Vladova *et al.* 2000) suggests that these proteins are probably universal (Misra and Green 1990). *A. cunninghamii* would seems to belong to the evolutionary scheme that connects most gymnosperms, while studies on the rbcL gene sequence support the hypothesis that *A. bidwillii* should not be placed at the bottom of the phylogenetic tree of the *Araucariaceae* but in the terminal cluster together with *A. hunsteinii* (Setoguchi *et al.* 1998). This data suggests that *A. bidwillii* may have a more recent origin than *A. cunninghamii*. Thus the atypical electrophoretic profile found in *A. bidwillii* might be the result of phylogenetic differences between the two species.

References

Allona, I., Casado, R., Aragoncillo, C.: Seed storage protein from *Pinus pinaster* Ait.: homology of major components with 11S proteins from angiosperms. - Plant Sci. **87**: 9-18, 1992.

Allona, I., Collada, C., Casado, R., Aragoncillo, C.: Electrophoretic analysis of seed storage proteins from gymnosperms. - Electrophoresis **15**: 1062-1067, 1994.

Bensadoun, A., Weinstein, D.: Assay of proteins in the presence of interfering materials. - Anal. Biochem. **70**: 241-250, 1976.

Capocchi, A., Cinollo, M., Galleschi, L., Saviozzi, F., Calucci, L., Pinzino, C., Zandomeneghi, M.: Degradation of gluten by proteases from dry and germinating wheat (*Triticum durum*) seeds: an *in vitro* approach to storage protein mobilization. - J. Agr. Food Chem. **48**: 5271-6279, 2000.

Del Zoppo, M., Galleschi, L., Onnis, A., Pardossi, A., Saviozzi, F.: Effect of salinity on water relation, sodium accumulation, chlorophyll content and proteolytic enzymes in a wild wheat.- Biol. Plant. **42**: 97-104, 1999.

Del Zoppo, M., Galleschi, L., Onnis, A., Saviozzi, F.: Salinity and proteolytic enzymes in germinating seeds of × *Haynaldoticum sardoum* (*Poaceae*). - Phyton **38**: 281-291, 1998a.

Del Zoppo, M., Galleschi, L., Saviozzi, F.: Long-term storage of *Araucaria bidwillii* Hook. seeds. - Seed Sci. Technol. **26**: 267-270, 1998b.

Doi, E., Komori, N., Matoba, T., Morita, Y.: Carboxypeptidase-like metal enzyme in rice seedlings. - Agr. biol. Chem. **44**: 921-922, 1980.

Florkin, M., Stotz, E.H.: Units of enzyme activity. - In: Florkin, M., Stotz, E.H. (ed.): Comprehensive Biochemistry. Vol. 13. Pp. 26-27. Elsevier, Amsterdam 1973.

Galleschi, L.: Proteolysis in germinating cereal grains. - Rec. Res. Develop. Phytochem. **2**: 95-106, 1998.

Galleschi, L., Capocchi. A., Giannoni. P., Floris, C.: Proteinase activities in quiescent and germinating seeds of × *Haynaldoticum sardoum.* - Physiol. Plant. **75**: 1-6. 1989.

Gifford, D.J.: An electrophoretic analysis of the seed proteins from *Pinus monticola* and eight other species of pine. - Can. J. Bot. **66**: 1808-1812, 1988.

Gifford, D.J., Greenwood, J.S., Bewley, D.J.: Deposition of matrix and crystalloid storage proteins during protein body development in the endosperm of *Ricinus communis* L. cv. Hale seeds. - Plant Physiol. **69**: 1471-1478, 1982.

Gifford, D.J., Tolley, C.: The seed proteins of white spruce and their mobilization following germination. - Physiol. Plant. **77**: 254-261, 1989.

Gifford, D.J., Wenzel, K.A., Lammer, D.L.: Lodgepole pine seed germination. I. Changes in peptidase activity in the megagametophyte and embryonic axis. - Can. J. Bot. **67**: 2539-2543, 1989.

Haider, A.S., El-Shanshoury, A.R.: Variability of storage proteins and esterase isozymes in *Vicia sativa* subspecies. - Biol. Plant. **43**: 205-209, 2000.

King, J.E., Gifford, D.J.: Amino acid utilization in seeds of loblolly pine during germination and early seedling growth. - Plant Physiol. **113**: 1125-1135, 1997.

Koenig, R., Stegemann, H., Franksen, H., Paul, H.L.: Protein subunits in the potato virus X group. Determination of the molecular weights by polyacrylamide electrophoresis. - Biochim. biophys. Acta **207**: 184-189, 1970.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Mikola, J.: Acid carboxypeptidases in grains and leaves of wheat, *Triticum aestivum* L. - Plant Physiol. **81**: 823-829, 1986.

Misra, S., Green, M.J.: Developmental gene expression in conifer embryogenesis and germination. I. Seed proteins and protein body composition of mature embryo and the megagametophyte of white spruce (*Picea glauca* Voss.). - Plant Sci. **68**: 163-173, 1990.

Roberts, E.H.: Predicting the storage life of seeds. - Seed Sci. Technol. **1**: 499-514, 1973.

Ryan, C.A., Walker-Simmons, M.: Plant proteinases. - In: Marcus, A. (ed.): The Biochemistry of Plants. Protein and Nucleic Acids. Vol. 6. Pp. 321-349. Academic Press, New York - London 1981.

Salmia,. M.A.: Proteinase activities in resting and germinating seeds of Scots pine, *Pinus sylvestris*. - Physiol. Plant. **53**: 39-47, 1981.

Salmia, M.A., Mikola, J.J.: Activities of two peptidases in resting and germinating seeds of Scots pine, *Pinus sylvestris*. - Physiol. Plant. **33**: 261-265, 1975.

Salmia, M.A., Mikola, J.J.: Localization and activity of a carboxypeptidase in germinating seeds of Scots pine, *Pinus sylvestris*. - Physiol. Plant. **36**: 388-392, 1976.

Sarath, G., De La Motte, R.S., Wagner, F.W.: Protease assay methods. - In: Beynon, R.J, Bond, J.S. (ed.) Proteolytic Enzymes: a Practical Approach. Pp. 25-55. IRL Press, Oxford 1989.

Setoguchi, H., Osawa, T.A., Pintaud, J.C., Jaffré, T., Veillon, J.M.: Phylogenetic relationships within *Araucariaceae* based on rbcL gene sequences. - Amer. J. Bot. **85**: 1507-1516, 1998.

Tormakangas, K., Kervinen, J., Ostman, A., Teeri, T.: Tissue-specific localisation of aspartic proteinase in developing and germinating barley grains. - Planta **195**: 116-125, 1994.

Vladova, R., Pandeva, R., Petcolicheva, K.: Seed storage proteins in *Capsicum annuum* cultivars. - Biol. Plant. **43**: 291-295, 2000.